NOAA Technical Memorandum NMFS-NWFSC-100

Zebrafish *(Danio rerio)* Husbandry and Colony Maintenance at the Northwest Fisheries Science Center

May 2009

U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
National Marine Fisheries Service

NOAA Technical Memorandum
NMFS-NWFSC Series
The Northwest Fisheries Science Center of the National Marine Fisheries Service, NOAA, uses the NOAA Technical Memorandum NMFS-NWFSC series to issue scientific and technical publications. Manuscripts have been peer reviewed and edited. Documents published in this series may be cited in the scientific and technical literature.

The NMFS-NWFSC Technical Memorandum series of the Northwest Fisheries Science Center continues the NMFS-F/NWC series established in 1970 by the Northwest & Alaska Fisheries Science Center, which has since been split into the Northwest Fisheries Science Center and the Alaska Fisheries Science Center. The NMFS-AFSC Technical Memorandum series is now being used by the Alaska Fisheries Science Center.

This document should be referenced as follows:
Linbo, T.L. 2009. Zebrafish *(Danio rerio)* husbandry and colony maintenance at the Northwest Fisheries Science Center. U.S. Dept. Commer., NOAA Tech. Memo. NMFS-NWFSC-100, 62 p.

NOAA Technical Memorandum NMFS-NWFSC-100

Zebrafish *(Danio rerio)* Husbandry and Colony Maintenance at the Northwest Fisheries Science Center

Tiffany L. Linbo

Northwest Fisheries Science Center
Environmental Conservation Division
2725 Montlake Boulevard East
Seattle, Washington 98112

May 2009

U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
National Marine Fisheries Service

Most NOAA Technical Memorandums NMFS-NWFSC are available online at the Northwest Fisheries Science Center web site (http://www.nwfsc.noaa.gov)

Copies are also available from:
National Technical Information Service
5285 Port Royal Road
Springfield, VA 22161
phone orders (1-800-553-6847)
e-mail orders (orders@ntis.fedworld.gov)

Table of Contents

List of Figures

Executive Summary

This technical memorandum describes the protocols and procedures for maintaining a small-scale zebrafish (*Danio rerio*) operation at the Northwest Fisheries Science Center, Environmental Conservation Division. Detailed descriptions are presented for Zebrafish Module maintenance, spawning, larvae rearing, adult feeding, quarantine procedures, and general fish health. These protocols may not be appropriate for all zebrafish system sizes and configurations; however, small zebrafish laboratories may find that this technical memorandum provides useful guidance for managing a healthy, breeding zebrafish colony.

Acknowledgments

This guide is a gathering of invaluable information and experience gained over many years of working, consulting, and troubleshooting with Laura Swaim and David White of the University of Washington Zebrafish Facility, Tor Linbo of the University of Washington Department of Biological Structure, and Carla Stehr, Megan Bushnell, and other colleagues of the Northwest Fisheries Science Center, Environmental Conservation Division. Laura Swaim and Carla Stehr developed and set up our system in 2001. Nat Scholz obtained funding through a Northwest Fisheries Science Center internal grant to establish the system. The information on general fish physiology and fish diseases was obtained from the Health Management of Laboratory Fish course at the Mount Desert Island Biological Lab in Maine.

Introduction

The Environmental Conservation Division of the Northwest Fisheries Science Center (NWFSC) has developed protocols to manage and maintain a small-scale zebrafish (*Danio rerio*) colony. Small-scale refers to less than 3,000 zebrafish adults. These protocols were specifically developed for the aquaculture system used by the NWFSC, a modified Zebrafish Module (Z-Mod) manufactured by Marine Biotech (Appendix A: Ordering Information).

Zebrafish are a useful research model because of their high year-round fecundity, wide array of molecular and genetic markers, rapid development, and relative ease of maintaining a breeding colony. Also as a teleost, the zebrafish may be used as an experimental model for other fish, such as salmonids. At the NWFSC, zebrafish are used to study the developmental, behavioral, and physiological effects of anthropogenic contaminants (e.g., polycyclic aromatic hydrocarbons, metals, pesticides, and estrogenic compounds), natural toxins (e.g., biotoxins from harmful algal blooms), and toxic bacteria (e.g., Vibrio). Since embryos, larvae, and adult fish are used in our studies, it is important to maintain healthy zebrafish throughout their lifecycle without introducing pollutants, chemicals, or antibiotics.

One of the most important aspects of keeping a healthy colony is providing consistent care to achieve a stable environment for the fish. Some of the protocols described here involve additional experience in recognizing the needs of the fish. For example, how much food to feed and prepare for fish requires firsthand experience to know when fish are satiated. Tank cleaning requires the ability to identify when bacterial growth could become a health hazard. A dedicated zebrafish caretaker with the ability to recognize and address the many zebrafish needs is vital to the overall health and productivity of the colony.

The products and supplies mentioned in this guide are routinely used for NWFSC's zebrafish husbandry. However, they may not be suitable for all zebrafish laboratories; therefore, information on product suppliers is not limited to what can be found in Appendix A: Ordering Information.

General Maintenance Overview

Daily (listed in the order the duties should be performed):

Morning:

- Clean larval tanks (see Cleaning) and feed larvae (see Raising Larvae and Beyond).
- Feed adult fish using salmon starter and *Artemia* sp. (brine shrimp) nauplii. Feed the dry food first (see Food for Adult Zebrafish).
- Take readings for ammonia, conductivity, pH, and temperature. Make adjustments if necessary (see Zebrafish Module Water Quality).
- Check sump water level. Add reverse osmotic and deionized (RO/DI) water if needed (see Water System).
- Record feeding and water quality measurements on daily logs.

Noon:

- Feed larvae (see Raising Larvae and Beyond).

Afternoon:

- Feed larvae (see Raising Larvae and Beyond).
- Feed adult fish salmon starter and *Artemia* (see Food for Adult Zebrafish).
- Take readings for pH and temperature. Make adjustments if necessary (see Zebrafish Module Water Quality).
- Prepare batch of *Artemia* for next day's feeding and start a new batch of *Artemia* (see Food for Adult Zebrafish).
- Record feeding and water quality measurements on daily logs (see Record Keeping).
- Check sump water level. Add more RO/DI water if water level has decreased due to evaporation (usually requires 3–4 gallons [gal] of RO/DI water per day) (see Water System).
- Refill the RO/DI water bucket. It will take about 90 minutes (min) to fill a five-gal bucket (see Water System).

Once a week:

- Measure nitrite levels in Z-Mod system (see Zebrafish Module Water Quality).
- Clean the tops of the tanks (remove old food debris) and make sure all water outlets are clear and not clogged with food debris (see Cleaning).
- Check to see if the shelves and back of the Z-Mod need to be cleaned (see Cleaning).
- Record all cleanings on log sheets (see Record Keeping).
- Flush out RO membrane filter (see Water System).
- Change Net Soak solution (see Cleaning).

Once every 2 weeks:

- Siphon debris out of tanks (see Cleaning).
- Clean sump tank (see Cleaning).
- Inoculate paramecium (*Paramecium multimicronucleatum*) bottle culture (see Paramecium Culture).
- Change and clean sump sock if necessary (see Cleaning).

Once a month:

- Check for chlorine and total dissolved solids (TDS) (see Zebrafish Module Water Quality).
- Calibrate pH meter and conductivity meters (refer to instructions in instruction manual folder).
- Change paper filter (#3) of Z-Mod. Replace carbon and crushed coral (see Filter Maintenance).
- Start new paramecium Tupperware culture (see Paramecium Culture).
- Change water in bleach bath (see Cleaning).

Once every 3 months:

- Clean biofilters (sponge filters #1 and #2) on Z-Mod (see Filter Maintenance).
- Set up spawns to maintain zebrafish colony (see Maintaining a Breeding Zebrafish Colony).
- Replace RO/DI system's sediment and carbon cartridges (see Water System).

Once a year:

- Replace UV bulb (see Filter Maintenance).
- Replace RO/DI deionizing filters (see Water System).
- Replace Ammonia Alert (see Zebrafish Module Water Quality).
- Order new adult and larval fish food (see Food for Adult Zebrafish).

Once every 2 years:

- Replace RO membrane (see Water System).

Water and Water Quality

Water System

Zebrafish systems require a controlled, clean source of water for basic facilities operations. Municipal water that has passed through a reverse osmotic and deionization (RO/DI) system is used to make system water, *Artemia* water, and for general maintenance in the zebrafish facility.

Reverse Osmotic and Deionized Water and the RO/DI System

RO/DI water is dispensed from the AquaFX's Mako 5-filter RO/DI system (Figure 1) mounted on the wall. The RO/DI water is stored in two 5-gal buckets labeled "RO/DI water only" (nothing else is put in these buckets).

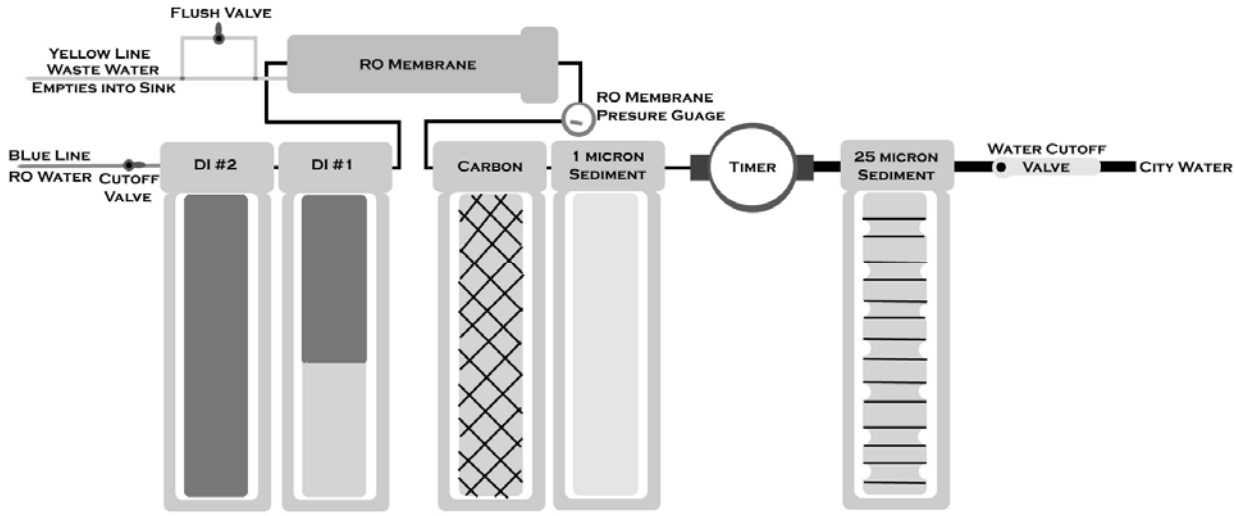

Figure 1. Schematic diagram of the RO/DI system. From the right, city water is filtered through a 25-micron sediment filter before entering the RO/DI system. A water timer controls the length of time the system runs. Water enters the system and travels through a 1-micron sediment and a carbon filter. It then travels through the RO membrane. Waste from the RO membrane is routed through the waste water line (yellow line) that should be directed into the sink. Water processed by the RO membrane continues through two DI filters and exits through the RO/DI water blue line. Two cutoff valves are located at the start and end of the system to allow for system maintenance. A flush valve is located on the waste water line to back flush the RO membrane for cleaning.

Before entering the RO/DI filter system, the city water is run through a 25-micron sediment filter, which is provided by NWFSC maintenance, to remove large particulates. Typically the filter is changed every 2–3 months. If there are problems with this filter (e.g., leaking), contact building maintenance.

After passing through the 25-micron filter, the water goes through a water timer, which controls the length of time that water passes through the RO/DI system. Water is first filtered through the sediment filters: a 1-micron filter and a carbon filter. It then goes through the RO membrane on top of the unit. Wastewater from the RO process goes out the yellow drain hose (that drains into the sink), while the RO water continues through the two deionizing filters. Finally, the RO/DI water comes out the blue hose and collects in 5-gal RO/DI buckets.

To turn on the RO/DI water system, turn the cutoff valve that comes out of the deionization filters so it parallels the blue tube, then open the main cutoff valve located before the 25-micron filter (Figure 1). Now turn on the water timer (located between the 25-micron filter and the RO/DI system). Normally, all valves are open and only the timer needs to be turned on. The timer can be set for up to 120 min (or it can be turned to manual, which will run indefinitely until turned to the off position. If it is turned to manual, remember to turn it off so the room does not flood. The RO/DI system is rated to produce RO/DI water at 100 gal per day. Actual use suggests that it produces just under 3 gal an hour (h) or 5 gal in 90 min.

Replace the two sediment filters every 2–3 months (depending on the season's influence on city water). Replace the RO membrane every 2 years. Replace one of the deionization filters once a year. The deionization filters are rotated through the two canisters, spending 1 year in each canister, so filters are used a total of 2 years. Specifically, remove and discard the first deionization filter from the first slot when is has changed color completely (from blue to orange over about 2 years) and replace it with the deionization filter that was in the second slot (after 1 year). Put a new deionization filter into the second slot (refer to AquaFX directions online at http://www.aquariumwaterfilters.com/library/manuals/5%20Stage%20Mako%20Manual.pdf).

A flush valve is located on the top of the RO/DI water filter system (Figure 1). The membrane should be flushed once a week or more, depending on RO/DI water usage. To flush the membrane, allow water to run through the RO/DI system and turn the blue valve on the flush valve kit so it parallels the white tubing. Water flow out of the blue hose (where the RO/DI water comes out) should stop, and water flow out of the yellow hose (the non-RO water) should increase. Let the water flush out the system for about 30 seconds (refer to AquaFX directions online at the URL above). To resume RO water filtering, turn the blue valve perpendicular to the white hose.

System Water

System water is RO/DI water amended with artificial sea salt (Instant Ocean, Appendix A) to a conductivity of 1,500–1,600 µS/cm and a pH of 7.0–8.0. It is stored in the 50-gal tower. System water is used in the Z-mod and for water changes, paramecium cultures, spawning, and other needs.

To prepare system water, first add RO/DI water to the system tower while keeping track of how much water has been added. Dissolve salt in a small amount of RO/DI water and add it to the tank at a rate of about 3 tbsp Instant Ocean salt per 20 gal RO/DI water, or 1 tbsp for every 2 h the RO/DI system is running. The tower is marked with a guide for the amount of sea salt to add when filling.

A submersible pump on the bottom of the tank is on 24 h a day to circulate the water. Check the pump periodically to make sure it is working. After adding salt, let the water stir for a while, then test the conductivity. It should be around 1,500 µS/cm. If below 1,400 µS/cm, add more salt. If above 1,600 µS/cm, add more RO/DI water.

Check the pH of the system water as well. RO/DI water has a pH of approximately 5.5, however system water should have a pH of 7.4–7.8. To increase the pH of freshly made system water, add sodium bicarbonate to the system water tower (approximately 100 ml of 1 molar sodium bicarbonate solution to 50 gal). This will also increase conductivity, so recheck the conductivity of the system water. Dilute with more RO/DI water if conductivity is too high.

Filling the system water tower can take an entire day, so prepare accordingly. A 5-gal bucket (labeled System Water) should be filled as a reserve before adding RO/DI water to the system water tower. The reserve system water in the bucket can be used while the tower is filled and the system water is adjusted for conductivity and pH.

Zebrafish Module Water Quality

Maintaining optimal water quality is vital for fish health. These water quality parameters are interconnected and a sudden change in one could signify a larger problem (Appendix B: General Fish Aquaculture Information). The following water quality parameters need to be monitored and kept stable.

Conductivity

Check daily. Conductivity should be 1,500–1,600 µS/cm.

If conductivity is too high, first check to see whether the water level in the sump is low. Water evaporation will lead to an increase in salt concentration. Add RO/DI water if needed and recheck the conductivity after several hours. If the conductivity is still too high, empty some of the water from the sump and replace it with RO/DI water. Give the system several hours to circulate and check the conductivity again.

If the conductivity is too low (this is usually not a problem), check the water level and add system water if needed. If the conductivity is still too low, dissolve a little salt (1 tbsp or less) in RO/DI water and add to it the sump. Give the system several hours to circulate and check the conductivity again.

pH

Check twice daily. The pH range of the Z-Mod should be between 7.0 and 8.0, but the optimum range is 7.0–7.4.

If it gets more acidic than 7.0 (the pH of the Z-Mod will naturally go down as ammonia- and nitrite-metabolizing bacteria lower the alkalinity of the water, Appendix B: General Fish Aquaculture Information), try gentle changes, such as adding crushed coral to the mesh bag in the filtration system (see Filter Maintenance) or replacing some system water to increase the pH. Sodium bicarbonate can also be added using a 1 molar stock solution of sodium bicarbonate dissolved in RO/DI water. Depending on how low the pH level is, start by adding 50 ml of the stock solution to the sump. However, do not add large amounts of sodium bicarbonate because sudden changes in pH could be stressful to the fish and overload the capabilities of the biofilter bacteria to convert ammonia (NH_3). Under acidic conditions, the biofilter cannot convert the nontoxic ammonium (NH_4+); instead, it can only convert NH_3 (Appendix B: General Fish Aquaculture Information), causing an accumulation of this ion. A sudden rise in pH, however, will cause the accumulated NH_4+ to shift to excessive amounts of toxic NH_3, which the biofilter may not be able to handle. Therefore, increases in pH should be kept to a rate at which the biofilter can convert the resulting NH_3.

If the pH is too basic (more than 8.0, which is unlikely to occur), try changing the system water or removing the crushed coral.

Temperature

Check twice daily. The temperature should be around 26ºC. Although zebrafish are hardy and can withstand typical fluctuations in room temperatures, two submersible heaters are kept in the sump to provide a consistent water temperature. It is ideal to keep the environment as stable as possible for the fish, biofilters, and water parameters (Appendix B: General Fish Aquaculture Information).

If the water temperature falls below 22ºC, check the heaters in the sump. Replace any that are not working or add a higher-powered heater to the sump. If the temperature rises above 30ºC, check the heaters in the sump. Make sure the heater temperature is not set too high. If the room temperature is also higher than normal (≈23–24ºC), contact Maintenance to check the air conditioning units and the ventilation ducts. In the meantime, fill the sump with RO/DI water, as the evaporation rate increases during higher temperatures.

Ammonia (NH_3)

Check daily. Check the Ammonia Alert disc (located in a Z-Mod tank without fish) to monitor ammonia levels. The disc should be yellow (safe). The disc can detect levels as low as 0.05 ppm. Ammonia levels 0.8 ppm or higher are toxic to the fish. The Ammonia Alert sensor is replaced once a year.

If the center disc changes color from yellow to light green (safe to alert), this indicates that ammonia is detected in the water and the population of ammonia- and nitrite-metabolizing bacteria in the biofilter may have been harmed (likely due to sudden changes in water quality). The fish can survive with this amount of ammonia, but system water changes are needed immediately to dilute the ammonia. Allow the bacteria on the biofilters to repopulate while constantly monitoring the ammonia and nitrite levels (check three times a day) and performing

system water changes (once a day change out 5–10% of the system water) until the biofilters are working again.

If ammonia levels increase to blue (alarm), then more corrective actions are need. Use products such as Ammo-Lock (Appendix A) to temporarily reduce ammonia levels. However, this is not a long-term solution. The biofilters need to be repopulated with ammonia-metabolizing bacteria. Reduce the amount of ammonia introduced to the system by feeding the fish only once per day and, if possible, remove some of the fish from the system. Continue monitoring the ammonia and nitrite levels and performing system water changes.

Nitrite (NO_2)

Check weekly. Follow the directions of the test kit to check the nitrite levels. Nitrite levels should be zero.

If nitrite is detected, a water change is necessary. Recheck the levels after water changes. If nitrite is still detected in the water, then the biofilters are not working properly. Treat the system the same as if the ammonia levels were too high (see above). Monitor the ammonia and nitrite levels and perform water changes until the biofilters are working again.

Chlorine

Check monthly. To check the level of chlorine in Z-Mod water, follow the directions in the chlorine test kit. The RO/DI system should be removing the chlorine, so no chlorine should be in the Z-Mod water. However if chlorine is present, then the RO/DI system is likely not functioning properly.

Chlorine is harmful to fish; corrective actions are required if chlorine is detected. If chlorine is in the water, add Genesis Chlorine Neutralizer at 2 drops/gal to neutralize the chlorine. For the Z-Mod system, use 40 drops for 20 gal. Check the RO/DI water for chlorine. If chlorine is detected, change the carbon filter in the RO/DI system.

Total Dissolved Solids

Check monthly. To check the level of TDS in the RO/DI water, follow the directions in the TDS test kit. The TDS of the RO/DI water should be 000–001, indicating that the RO/DI system is working properly.

If dissolved solids are detected in the RO/DI water, check the RO/DI water filters. Check the gauge to make sure the water is being filtered within the operating range. If necessary, change RO/DI water cartridges (usually changing the sediment filters will correct the problem).

Sump Water Level

Check daily. Water level in the sump should be near the level marked by the tape that reads “maintain water level here,” located on the outside of the sump. The water level needs to be above the outlet to the water pump and 2–3 inches below the lip of the filter bag or sump sock (Figure 2).

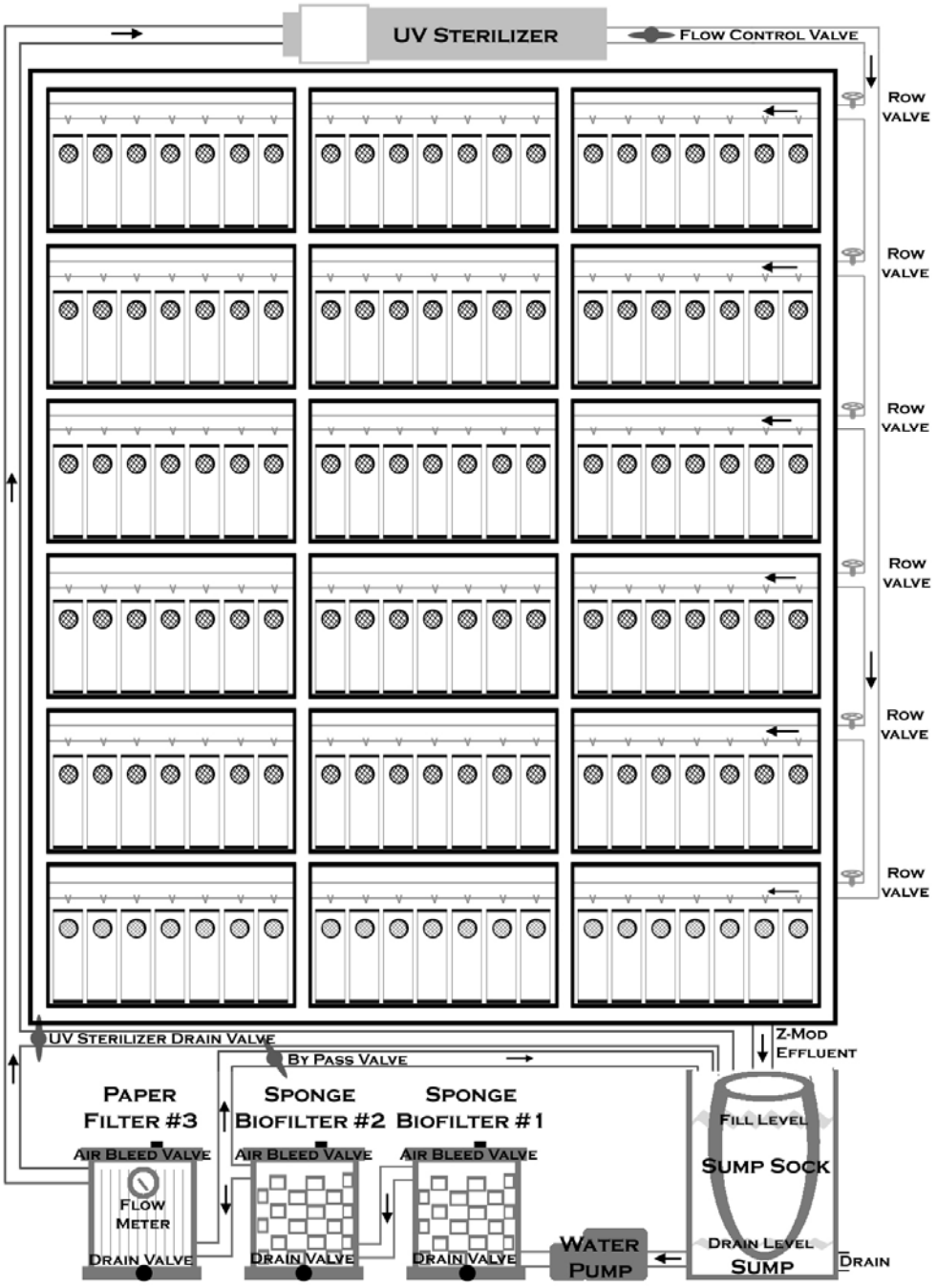

Figure 2. Schematic diagram of the self-contained, modified Z-Mod system. Arrows indicate the directional flow of the water. Water leaving the Z-Mod tanks (effluent) enters the sump, where it is filtered through the filter bag or sump sock to remove larger debris. The water pump powers the water flow through a modified three-step filtration system. Water passes through two sponge biofilters then through the paper filter, which also contains activated carbon (for chemical removal) and coral (for pH balance). Finally the water goes through a UV sterilizer and enters the Z-Mod tanks again. Individual row valves control water flow into the rows. The line on the sump indicates where the water level should be maintained. The bottom line on the sump indicates how low the water level is drained when doing maintenance (the water level should be kept above the drain to the water pump).

If water is low due to evaporation, add RO/DI water. When water evaporates, the salt stays in the system, so do not add system water or the conductivity will be too high. The normal evaporation rate is 3–4 gal a day.

Flow Meter and Bypass Valve

Check once per week and before filter maintenance. The flow meter should read approximately 5–7 gal per min.

The water flow of the Z-Mod system is a dynamic balance between the individual row valves and the bypass valve. Water flowing to each row can be adjusted by turning the red valves at the end of each row. To adjust the entire flow pressure, adjustments can be made to the bypass valve (white ball valve) that is closest to the sump on the PVC piping (Figure 2). The bypass valve alleviates backpressure on the sponge biofilters (see Filter Maintenance) and affects the pressure of the water entering the tanks. As more tanks are added to the Z-Mod, the pressure may need to be increased (i.e., the bypass value almost closed) to compensate for the additional rows being used. During sponge biofilter maintenance, however, it is important to open the bypass valve to relieve the initial water pressure when water reenters the filter canisters (see Filter Maintenance).

Zebrafish Module System Maintenance

Filter Maintenance

Since the Z-Mod is a closed system (water recirculates within the system), the water entering the tanks must first pass through a filtration system to remove particulates, ammonia, and chemicals. The modified filter system (Figure 2), which is not the Z-Mod filtration system offered by Marine Biotech, consists of a sump (or a reservoir of water), a water pump that circulates water through two sponge biofilters, a paper filter with carbon and coral, and the UV sterilizer. To remove large debris (e.g., uneaten food, bacterial growth) and escaped fish, Z-Mod effluent is first filtered through a filter bag or sump sock as it enters the sump. Then the water is pumped through two sponge biofilters (#1 and #2), which remove ammonia and nitrite. Next the water goes through the paper filter (#3) for fine particle removal, pH balance (coral), and chemical removal (activated carbon). For the final recirculation step, the water passes through a UV light sterilizer, then the filtered water reenters the Z-Mod tanks.

Maintenance on the Z-Mod is preferably scheduled earlier in the day. If canisters are not tightened properly, a leak can be seen and corrected later in the day. It is better to catch the problem earlier (on the same day) than later (the next morning after the Z-Mod has been running and leaking overnight).

Filter Bag or Sump Sock

Clean every 2–3 weeks or as needed (when the water level in the filter bag is higher than the water level of the sump or when the bag is overflowing). Remove the felt filter bag or sump sock and quickly exchange it for a clean one. Use the tap water hose to blast debris off the used sump sock. Soak the sump sock in a 5% bleach solution (100 ml/5 gal or ≈250 ppm active chlorine) overnight. It may need two nights of 5% bleaching to get rid of the grime. The next morning, rinse the sump sock with tap water for a few minutes. Soak the sock in clean water for several hours. Rinse again in running water and hang dry.

Paper Filter

Clean once a month or when pressure is less than 5 pounds per square inch or flow rate has dropped. Turn off the pump. Attach the hose to the sump and drain the sump water down to the green tape mark. Open the air bleed valve on the filter casing (white valve on top of the lid). Attach the hose to the drain valve on the Ocean Clear (Appendix A) filter casing #3, and open the valve to drain water from the filter (the other filters will drain part way down as well). Unscrew the lid (use the rubber mallet to help loosen the lid).

Remove the carbon in the center and throw it away, keeping the mesh bag. Remove the coral bag, but do not throw it away. Put the coral in a "used coral" container (to be reautoclaved)

and rinse out the bag. Reused crushed coral is good for up to 6 months. Remove the used circular paper filter.

Rinse the filter casing (using RO/DI or system water) and use paper towels to remove bacterial growth inside the case. Let the dirty water drain from the valve. When the water has completely drained, remove the hose and close the valve on the casing.

Place a clean paper filter in the casing. Fill the carbon bag with about 3–4 cups of new Kent Marine Reef Carbon. Rinse the carbon with RO/DI water. Add ½ to 1 cup of autoclaved coral to the coral bag. Put the carbon and coral bags inside the circular filter.

Wipe down the O-ring and lid of the Ocean Clear case with a paper towel. Put a small amount of silicon grease (safe for fish) on the O-ring and screw the lid on the filter. Use the rubber mallet to help tighten the lid (do not over tighten).

Fill the sump with system water. Have extra water in a 5-gal bucket to top off the sump as water circulates back into the filters. Turn on the pump. When water reaches the top of the paper filter #3, it will come out the air bleed valve. Close the air bleed valve. Air will slowly continue to bubble and build up at the top of the canister case. Open the air bleed valve to remove this air.

Clean the dirty paper filter by rinsing it with warm tap water. Put the soiled filter in a 5-gal bucket. Let it sit in a 5% bleach solution (100 ml bleach/5 gal tap water) overnight. Next day refresh the 5% bleach bath for a second night of soaking. On the third day, rinse the filter with tap water. Add new tap water and 30 g of sodium thiosulfate to chelate the bleach left in the filter. Soak overnight. Rinse completely with tap water and air dry.

Make sure to log all maintenance information in the logbook (Appendix C: Examples of Zebrafish Records).

Sponge Biofilters

Sponge biofilters serve an important role in maintaining the nitrogen cycle (Figure 3 and Appendix B). The biofilter provides a large surface area for the ammonia- and nitrite-metabolizing bacteria (our biofilters are polystrand dual filter pads). Sudden changes in the water quality or chemistry (e.g., pH or the presence of chlorine) can kill the bacteria and stop the nitrogen cycle, which will lead to the build up of harmful ammonia and nitrites. Therefore it is important to keep water conditions stable for the bacteria and ultimately the fish.

Clean biofilters every 3 months. Turn off the water pump. Attach the hose to the sump valve and drain the water in sump to the green tape (above the drain to the pump). Open the air bleed valve (white valve on top of the lid) on Ocean Clear filter casing #1. Attach the hose to the drain valve on Ocean Clear filter casing #1, open valve and drain water from the filter (the other filters will also drain part way down). Unscrew the filter lid (if necessary, use the rubber mallet to help loosen it).

Transfer the sponge pads from the filter case and place them in a bucket of clean system water so they do not dry out. Using RO/DI or system water, rinse down and use paper towels to

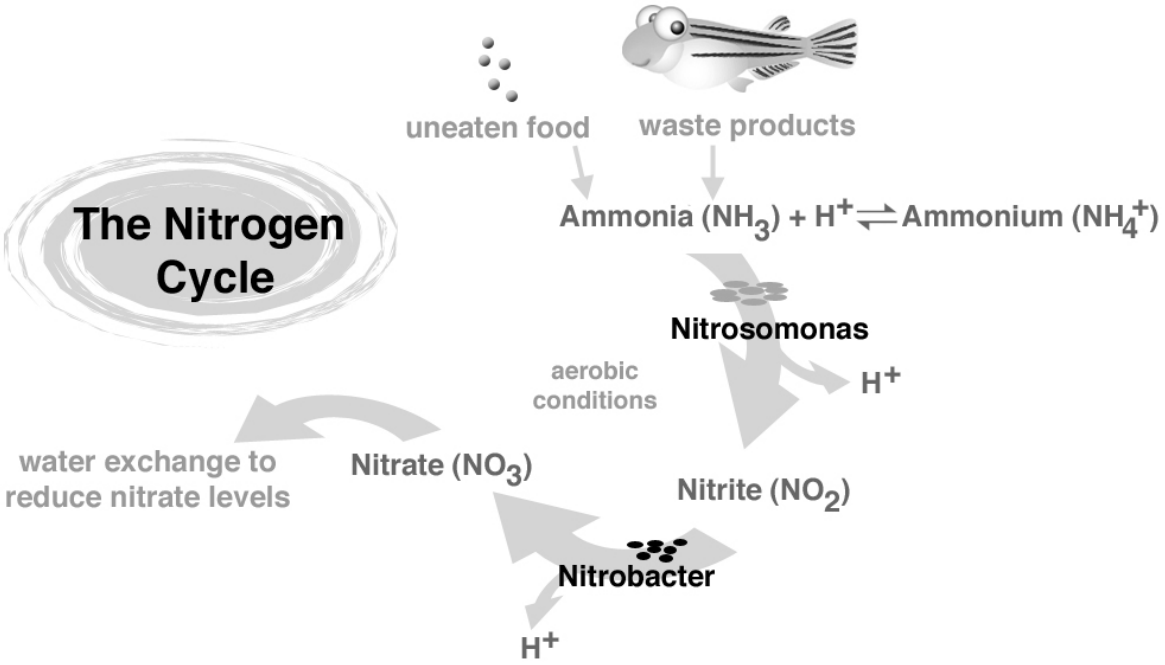

Figure 3. The nitrogen cycle in aquaculture systems and aquarium tanks. Ammonia is built up from fish excretion and uneaten food. *Nitrosomonas*, a bacterium, converts the ammonia into nitrite, which is converted into nitrate by *nitrobacter*, another bacterium. During both chemical conversions by the bacteria, H^+ is released causing a reduction in pH. A reduced pH will change ammonia into ammonium, which cannot be converted by the bacteria. Ammonia and nitrite are toxic to fish; nitrate is also toxic to fish, but at a much higher level. To reduce nitrate levels, routine water changes are required.

remove bacterial growth inside the filter case. Let the dirty water drain out of the valve. Remove the hose and close the valve once the water has drained. Wring excess bacteria from the filter pads in the bucket of system water. Removing the excess bacterial growth will allow water to flow through the filters efficiently, while the remaining population of ammonia-metabolizing and nitrite-metabolizing bacteria will continue the nitrogen cycle (Figure 3).

Wipe down the O-ring on top of the canister and the canister lid with a paper towel. Apply silicon lubricant in a light layer on the filter casing's O-ring. Screw on the lid. Use the rubber mallet to help tighten the lid.

Repeat the process with sponge filter #2.

Fill the sump with system water so the pump does not go dry when it is turned back on. Keep buckets of water nearby to keep the sump full as the water refills the filters. Check to make sure the bypass valve is open.

Turn on the water pump and close the air bleed valves on filter casings #1 and #2 when the water reaches the top of the filter and starts to come out of the air bleed valves.

Readjust water flow into the tanks via individual row valves and the bypass valve if necessary (see Flow Meter and Bypass Valve).

Log all maintenance information in the logbook (Appendix C: Examples of Zebrafish Records).

UV Light Maintenance

A UV sterilizer kills pathogens before the water enters the fish tanks and is essential in preventing the spread of disease within the zebrafish colony. The efficacy of the sterilization process depends on keeping the unit well maintained. The UV sterilizer (Emperor Aquatics Smart High Output UV Sterilizer) should be on at all times (except when replacing the bulb). The bulb should be replaced every 12 months to ensure the effectiveness of the sterilization process.

Consult the UV sterilizer manual (online at http://www.emperoraquatics.com/SMARTUV_Instructions_07.pdf) for full instructions and diagrams. Four feet of clearance is needed to remove the bulb. Unplug the light before replacing the bulb. Drain the water from the sump, keeping the water level above the outlet to the water pump. To drain water from UV unit, open the valve to the UV sterilizer drain (it is the ball valve above the pump, towards the back of the Z-Mod, on the tubing). Tilt the UV unit and drain the water out. A little bit of water will remain and drip out when the unit is opened, so be prepared for some water to leak. Unscrew the end (black retaining nut) of the UV light. Unscrew the internal white quartz sleeve retaining nut (it holds the quartz sleeve to the ballast). Unplug the ballast from the UV light. Carefully remove the bulb from the quartz sleeve.

Check to see whether the quartz sleeve is clear. If there is any opacity, clean it off with a scrub brush and a little water; opacities on the sleeve decrease the efficacy of the bulb. Slide a new UV bulb into the quartz sleeve. Plug the ballast back into the UV light. Hand tighten the white internal and black external retaining nuts into place, making sure they are well sealed. Turn the UV light back on. Close the ball valve to the UV sterilizer drain.

Fill the sump with RO/DI or system water. Turn on the pump to start water circulating into the UV light. Add more RO/DI or system water to the sump as water circulates through the UV sterilizer.

Leave the flow control valve by the UV light open (i.e., do not adjust it). It affects how fast the water goes through the UV system, which in turn affects the length of time the water is in contact with the UV light. In some circumstances (if a disease outbreak occurs) it may be desirable to partially close the valve to increase the time the water is exposed to the UV light. However, partially closing the valve also increases water pressure against the water pump. This will in turn add unwanted pressure throughout the filtration system.

Cleaning

Zebrafish equipment and the Z-Mod need constant cleaning to reduce chances of spreading disease among the fish and to reduce harmful levels of nitrates (Appendix D: Zebrafish Diseases).

It is important to note that the only disinfectants used are alcohols, bleach, benzalkonium chloride, and methylene blue. Items cleaned with alcohols and bleach should be completely dry before use. Benzalkonium chloride and methylene blue are the active ingredients in Net Soak (see Nets) and are not harmful to fish at the concentrations used. Detergents such as hand or dish soap should never be used on any zebrafish equipment. However, hand soap may be used to prevent the spread of diseases. Hand washing is encouraged before and after feeding or handling the fish.

Countertops

Countertops should be sterilized with 70% ethanol or isopropanol after fish tanks have been on the counter and before making paramecium cultures. This will reduce the spread of diseases among fish.

Also, no chemicals such as toxins, contaminants, or fixatives are allowed on the countertops in the zebrafish room, especially near the Z-Mod. Laboratory experiments utilizing zebrafish embryos, larva, or adults must take place away from the Z-Mod with no chance of introducing a chemical to the system. Exposed fish are not allowed to return to the zebrafish colony unless all traces of the exposure chemical are removed (and cannot be excreted by fish or transmitted with the fish water).

Nets

Use a clean net for each Z-Mod tank (i.e., do not double dip). Clean, dry nets are located on the drying rack.

Sterilize nets between each use. After using a net, rinse it with tap water. Place the net into the designated beaker of Net Soak (Appendix A) solution (≈1.5 tsps/5 L). Soak the net overnight. The following morning, remove the net from the Net Soak solution, shake off excess solution into the sink, and hang up the clean net on the drying rack. The Net Soak solution can also be used to sterilize sponges and bottlebrushes. Change the solution once per week or more often when use is heavy.

Bleach Bath

Use the bleach bath to sterilize spawning cages and other equipment that will come in contact with the fish (e.g., turkey basters, larval tanks, Z-Mod tanks, tank lids) or any cultured organism (e.g., paramecium Tupperware, paramecium beakers). Mostly glass and plastic items will be placed in the bleach bath. Use tap water to rinse and scrub off feces, old food, etc., from tanks and lids before fully submersing items into the bleach bath. Do not put turkey baster bulbs in the bath as the bleach will break down the rubber.

Change bleach bath water every 2–3 months or sooner if use is heavy. To make the bleach bath, add 750 ml of bleach to 10 gal of tap water (20% bleach solution or ≈1,000 ppm active chlorine). Leave items in the bleach bath for at least 1 h or overnight. Triple rinse the items with plenty of warm tap water in the sink. Air dry the items separately on the drying rack. Bleach can kill fish and embryos, so it is very important to rinse well and completely air dry.

Tank Lids and Outlets

Clean lids once a week or sooner if dirty. Using a paper towel or sterilized bottlebrush, wipe away food left on tank lid. If the tank lid is particularly dirty, switch out the dirty lid with a clean one. Scrub the dirty lid and put it in the bleach bath.

Check the outlet where the water flows out of the tank daily. It should be clear of debris. If bacteria and gunk are growing on the outlet mesh, use a finger or a sterilized bottlebrush to remove the material from the mesh (and out of the tank) or replace the dirty outlet with a clean one. Scrub the dirty outlet and put it in the bleach bath.

Nursery Tanks

Check nursery tanks (bottom row of the Z-Mod) daily and clean as needed. It is easy to overfeed fish in the nursery tanks, so check daily to make sure fungus is not growing on the bottom of the tanks.

Every other day the dirty tanks should be removed from the Z-Mod and fungus on the bottom of the tank taken out. Use a turkey baster to gently remove the fungus, being careful not aspirate small fish. Do not use a siphon to clean the nursery tanks, as the water flow would be too great for the small fish and would likely aspirate them.

Siphoning Adult Tanks

Check adult tanks for build up of debris on the bottom and clean with a siphon when dirty. Fuzzy buildup on the tank bottom is fungus growing on old food; the darker materials are feces. These can be harmful to zebrafish and should be siphoned out of the tank. To clean the tanks, remove the tanks to be siphoned from the Z-Mod and place them on the counter. It is easier to set several tanks on the counter at the same time.

The siphon tool (Figure 4) is a vacuum-shaped nozzle (a Nalgene T-type connector with the length of the top of the "T" cut off to form a small slot opening and aquarium safe silicon sealing the ends of the top of the "T") attached to a rigid tube. The other end of the rigid tube is connected to a flexible length of tubing with an inner diameter of 3/8 inch. A tubing pinch clamp is located on the flexible part of the tubing close to the rigid tube.

To siphon, fill the tubing with water (use system water in case there is back flow) by submerging the siphon tool in system water or pouring it through the tube. Once there is water in the siphon (with no large air bubbles), use the clamp on the tubing to pinch off the flow so the water cannot escape. Then place the nozzle in the tank and put the tube end in an empty bucket on the floor. Release the clamp and the water should start flowing into the bucket.

The water will flow from the high spot (the tank on the counter) to the lower spot (the bucket on the floor). Siphon out debris by moving the nozzle over the bottom of the tank, scraping, and loosening the debris to be siphoned off by the tube. To move from tank to tank without starting the siphoning process again, simply clamp the tubing and move the nozzle.

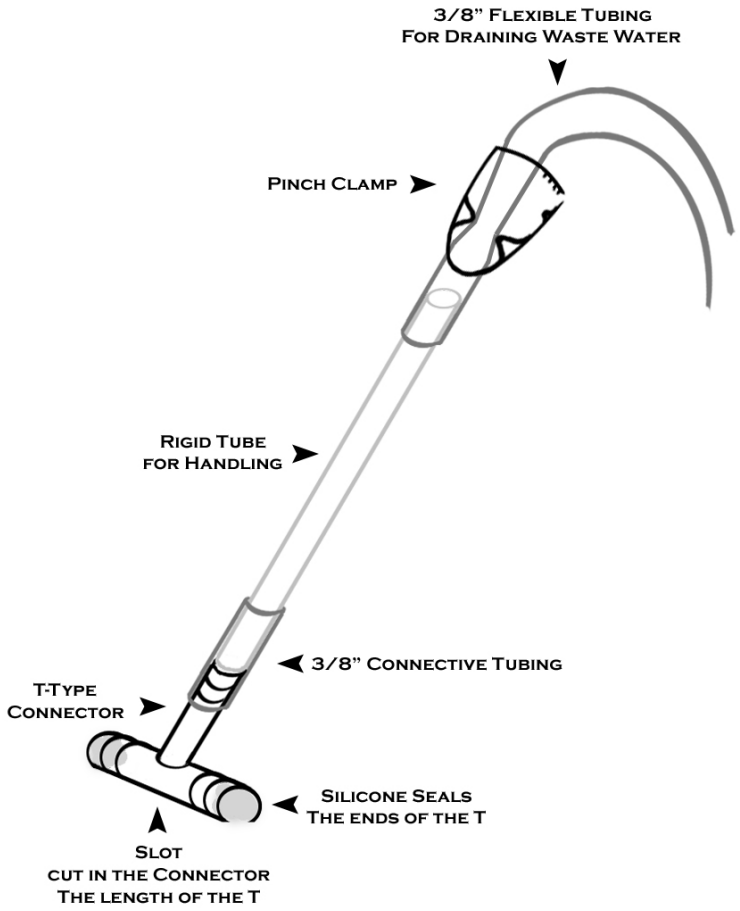

Figure 4. Siphon for cleaning out Z-Mod tanks. This easily assembled siphon makes removing detritus and feces from Z-Mod tanks more efficient.

Be careful not to siphon out all the water (there will still be fish in the tank during this process) or fish (smaller fish might get drawn into the siphon). Always check the bucket for fish before siphoning a different tank and before emptying the bucket.

Back of the Z-Mod

As part of its design, effluent tank water runs down the back, inside wall of the Z-Mod. Bacteria build up on the inside wall, making it brown and slimy. Once a year, use RO/DI or system water (not tap water) to clean a portion of the Z-Mod. Do not clean the entire Z-Mod at the same time or the sump sock will quickly become clogged with bacterial mats. Use a sponge that has not been treated with a fungicide (if a new sponge feels damp coming out of the packet, it has probably been treated with a fungicide), a sponge that has been well rinsed of the fungicide (i.e., the sponge is held under running tap water, while being repeatedly wrung out for 5–10 min), or paper towels. After cleaning the back of the Z-Mod, change the sump sock (see Filter Maintenance).

Culturing Zebrafish

Food for Adult Zebrafish

Adult zebrafish are fed dry salmon starter meal (Bio-Oregon Bio Vita starter, Appendix A) and *Artemia* sp. twice a day. Feed the dry salmon meal first, as it is more nutritious than the *Artemia*.

Dry Salmon Food

One pinch (0.05–0.07 g) of dry salmon meal is fed to each tank (of ≈15 fish). The salmon starter should be kept dry and well preserved; it is stored in a –20°C freezer. In order to ensure the zebrafish are given the right nutrients and vitamins (which degrade with time), new food should be ordered every year.

Since our zebrafish are used for toxicology research, each new bag of salmon starter should be tested in-house for contaminants (e.g., polycyclic aromatic hydrocarbons, persistent organic pollutants, and metals) to determine background levels and whether the food is suitable for use. These precautions are taken because fish processed by commercial fish food makers to make salmon meal could contain these pollutants.

Artemia Culture

Adult zebrafish are fed *Artemia* that are approximately 40–48-h old. Therefore, *Artemia* cultures will be fed to fish 2 days after they have been started (e.g., a culture started Monday afternoon will be fed Wednesday morning and afternoon).

Artemia culture setup

To hatch a small amount (at most 2 tsps) of *Artemia*, two 2-L *Artemia* cones are needed. One cone is designated the "0–24 hour" *Artemia*; the other is "24–48 hour" *Artemia*. An air pump with one air hose, which consists of aquarium tubing and a 1-ml glass serological pipette, is used for each cone to keep the *Artemia* eggs (cysts) well aerated and suspended in the water. Both cones of *Artemia* must be aerated at all times except when harvesting or preparing the *Artemia*.

Artemia hatch at around 20 h at 25ºC. However, at room temperature (23ºC) the hatching rate will be slightly delayed. Therefore, we culture our *Artemia* for more than 24 h. The additional time will allow any delayed *Artemia* cysts to hatch. Additionally, the two-cone system allows for more of the eggshells to be removed from the culture. While using a heater to increase the temperature of the *Artemia* culture will cause them to hatch sooner (see Starting Larger Amounts of *Artemia* Cysts), our two-cone method of hatching *Artemia* at room temperature meets our needs for a small zebrafish colony.

Feeding *Artemia* to the fish

To harvest the *Artemia*, remove the air hose from the 24–48-h cone. This cone contains *Artemia* that have been growing for nearly 2 days. After approximately 5 min, the *Artemia* culture will settle into three layers. Most of the unhatched cysts will settle to the bottom, the hatched *Artemia* will settle just above the bottom layer of unhatched eggs, and the eggshells will float to the top. To dispose of the unhatched eggs, open the bottom valve and release a small amount of brown liquid (containing the unhatched cysts) down the sink drain. Close the valve as soon as the water turns orange (containing *Artemia*).

To collect *Artemia* for feeding, first dampen the designated *Artemia* net with system water to allow the *Artemia* water to pass through more easily. For the first feeding of the day (in the morning), open the valve to drain one-half of the *Artemia* into the *Artemia* net. Return the remaining one-half of the *Artemia* in the 24–48-h funnel to the holder and put the air hose back in the funnel.

Rinse off excess salt water from *Artemia* in the net with a squirt bottle of system water. Turn the net inside out and rinse the *Artemia* off the net into a 100-ml glass evaporation bowl or similar size beaker using the squirt bottle of system water. Dilute the *Artemia* out to approximately 100 ml (if 2 tsp of *Artemia* cysts were hatched). Allow the *Artemia* to settle for 1–2 min. Any unhatched *Artemia* that have made it through the cone will settle on the bottom and the eggshells will form a brown layer floating on top of the bowl. When pipetting out the *Artemia*, avoid the eggs and shells as much as possible. Feeding the indigestible unhatched cysts and eggshells to fish, young ones in particular, can lead to gut obstruction and ultimately death. Use a pipette (plastic or glass, depending on preference) to remove *Artemia* from the finger bowl, and distribute them among the fish tanks. Feed according to the number of fish per tank (i.e., feed more if there are more fish in a tank, less if there are fewer). Feed approximately 1 ml of *Artemia* to a tank of 15 fish.

For the second, afternoon feeding, drain the remaining *Artemia* through the dampened *Artemia* net and close the valve before draining the eggshells on top. Collect the *Artemia* into the evaporation bowl and feed them to the zebrafish as described above. After the second feeding of the day, empty and scrub the cone using tap water and a sponge (see sponge comments under Cleaning the Back of the Z-Mod). The clean cone will be used to hold *Artemia* for the next day's feeding.

Preparing 0–24-h *Artemia* for next day's feeding

Every afternoon, after the second feeding or single feedings on the weekends, *Artemia* are prepared for the next day's feeding.

Remove the air hose from the 0–24-h *Artemia* cone, which contains *Artemia* that have been culturing for 24 h. The unhatched eggs and the hatched *Artemia* will settle to the bottom and the eggshells will float to the top. It is important not to transfer the majority of the eggshells at this stage. Open the bottom valve and drain the unhatched eggs and *Artemia* from the 0–24-h cone into the cleaned 24–48-h cone, but do not drain the eggshells floating on top into the 24–48-h cone. Close the bottom valve to stop the flow before reaching the eggshells on the top.

Artemia are able to start feeding at 32 h. Since the *Artemia* are fed to the zebrafish at 40–48 h, the *Artemia* are fed an enrichment to load their guts (bioencapsulation) to maintain their nutritional value. Add a couple dashes of *Artemia* food (a dry powder food mixture containing carotenoids and highly unsaturated fatty acids or a commercially available *Artemia* enrichment) to the 24–48-h *Artemia* cone. Replace the air hose in the 24–48-h cone to aerate the *Artemia* for next day's feeding.

Discard the eggshells from the 0–24-h cone. Rinse and scrub the cone under tap water with a sponge (see sponge comments under Cleaning the Back of the Z-Mod) to start a new *Artemia* culture. Bleach and dry the cones every 2 weeks to reduce bacterial growth, which will cause the eggs to stick to the sides of the cone (the eggs will not hatch).

Starting *Artemia* from cysts

A new batch of *Artemia* is made every afternoon.

Artemia require a salinity of about 1 tbsp of sea salt (Instant Ocean) per 1 L of RO/DI water (about 4 tbsp/gal). In the 2-L plastic container labeled "*Artemia* Water," add about 2 tbsp of sea salt to 1.75 L of RO/DI water. The solution should be saturated, so there will be some nondissolved salt on the bottom of the container. Decant the saltwater into the clean 0–24-h cone (try not to add the nondissolved salt to the *Artemia* water). Do not overfill the funnel above the fill line. If the water level is too high, *Artemia* cysts will stick to the lid of the funnel and will not hatch.

For our system containing approximately 2,000 fish, add about 2 tsp of *Artemia* cysts to the 0–24-h funnel filled with the *Artemia* water. The amount of eggs used may change depending on the number of fish in the breeding colony, but in general use about 1 tsp of eggs for 1,000 fish. Replace the air hose in the cone, and make sure the airflow is sufficient for the cysts to stay suspended in the water (the best position for the hose is on the bottom side of the cone). The *Artemia* will go anoxic and not hatch if aeration is not sufficient.

Artemia cysts are stored in the refrigerator (4ºC).

Starting larger amounts of *Artemia* cysts

If more than 2 tsp of *Artemia* are required, then additional hatching cones or a larger *Artemia* hatchery are needed. The volume of water should be scaled up at the same salinity, and the egg density (1 tsp cysts/1 L *Artemia* water) should be approximately the same or lower. If the density of the cysts is too high, the hatching rate will decrease.

To hatch a larger amount of cysts, eggs should first be decapsulated (see following subsection). Add an aquarium heater set to 26–28°C to the water so the *Artemia* will hatch at a faster rate. This way the *Artemia* will be ready to feed to the zebrafish within 24 h (ideally *Artemia* should be fed to the adult or larval zebrafish immediately after hatching). Harvest the *Artemia* within 24 h using the same protocols as above. The *Artemia* can be diluted into a small squirt bottle with system water for quicker feeding.

Starting *Artemia* using decapsulated cysts

When a larger amount of *Artemia* is required to feed a bigger zebrafish colony, *Artemia* cysts need to be decapsulated. The advantages of decapsulated eggs are a higher hatching rate and the softened eggshells are easier on the digestive systems of the both adult and larval zebrafish. However, the process of decapsulating cysts requires greater effort. When hatching smaller batches of cysts (see Starting *Artemia* from Cysts), the procedure described above is sufficient for removing indigestible eggs without taking additional time.

To decapsulate the eggs, add several teaspoons of cysts to a dish with system water. Let the eggs soak for at least 30 min. Pour the eggs into the *Artemia* net for cysts. Immerse the net with the eggs into a bowl of full strength bleach, while keeping the eggs contained in the net. Using a disposable pipette, stir the eggs until they start to change color from brown to a pale orange color (≈1.5 min). Rinse the eggs (still in the net) thoroughly with RO/DI water (pour at least 1 gal over the eggs) to remove all the chlorine (trace amounts of chlorine will kill the hatched *Artemia*). Add eggs to the hatchery cone filled with *Artemia* water, then add the air hose and ensure there is enough air movement to keep the eggs suspended.

Weekend Feedings

On weekends, when there are no larval fish in the husbandry facility, adult fish are fed once a day. Under these circumstances, weekend feedings are like afternoon feedings. The fish are fed dry salmon food and all of the *Artemia* in the 24–48-h cone are drained and fed. The 0–24-h *Artemia* are drained into an emptied and cleaned 24–48-h cone and a new *Artemia* culture is started in a cleaned 0–24-h cone. If larval fish are being raised over the weekend, then two feedings per day are required (see Raising Larvae and Beyond).

Spawning Fish and Collecting Embryos

The day before zebrafish embryos are needed, adult males and females are combined in spawning tanks in the afternoon. They are left overnight in the tanks together. Dawn (i.e., when the lights turn on in the morning) cues the fish to start spawning, which generally occurs during the first 4 h of the day. After spawning, the fish are returned to their respective Z-Mod tanks, and the embryos are collected, cleaned, and stored in the incubator.

Spawning fish

Set up fish to be spawned in the afternoon, before the last feeding of the day. This reduces the amount of feces on the bottom of the cage with the eggs (thus making the embryos easier to clean).

Before setting up the spawning tanks, sterilize the counter with 70% alcohol prior to fish transfer (if a fish falls onto the countertop, it does not get contaminated). To set up a spawn, fill the spawning tank (which consists of a plastic mesh bottom insert that fits into an outer plastic tank) with system water from the system water tower. Once the spawning tanks are filled with water, place them on the counter where the spawning will take place.

Select the tanks containing fish to be spawned from the Z-Mod. Fish can be spawned at most once per week, otherwise protein and lipid reserves in the fish become exhausted, resulting in a poor egg yield. Check the logbook to choose fish that have not been spawned within the last week.

Use nets that have been sterilized with purple Net Soak solution (see Nets) to transfer fish from their original tanks into the spawning tanks, using a different net for each tank. Depending on the number of adult fish available and the number of eggs needed, set up the spawns using the following ratios. To yield the greatest amount of eggs, the best spawning ratio is 2:3, males to females. Ratios of 1:1 or 1:2 also work (and are also less stressful for all fish involved).

In general, wild-type zebrafish can be sexed by color and body shape. Females have large bellies that descend from the body in the anterior region. Males have a red-yellowish hue in the anal and caudal fins and tail, and are more streamlined in shape (i.e., they lack a belly). Sexing fish takes practice and is greatly aided by direct guidance from someone who is experienced.

After placing males and females in the spawning tanks, clean the nets by rinsing out debris with tap water, then place them into Net Soak. Put covers on the spawning cages and stack the cages on the sterilized counter away from general use.

Label the spawning tanks with the fish tank names (see Maintaining a Breeding Zebrafish Colony). This ensures the fish are returned to their correct tank and avoids mix-ups.

The lights in the room are programmed to turn on at 800 hours and go off at 2200 (14-h light cycle). The next morning, zebrafish spawning behavior will be triggered by the lights coming on (simulates dawn). The fish will generally spawn during the first 4 h after the lights come on (between 800 and 1200). Occasionally, the fish will spawn the same evening the tanks are set up. This can be avoided by setting up the fish later in the day, after noon at the earliest.

To reduce the amount of feces mixed with the eggs and to stimulate spawning, move the inner tank with the fish to a fresh tank of system water early in the morning before spawning occurs.

Once the fish have spawned, they cease spawning behavior (males pursuing females). Record the spawning activity (which fish tanks were spawned, egg quality and quantity, time spawned) on the log sheets (Appendix C: Examples of Zebrafish Records). Put the fish back into their correct respective tanks using a clean net. Place them back on the Z-Mod to allow them to recover.

Embryo Collection

To harvest the embryos, remove the insert from the spawning tank. Once the embryos and feces have settled to the bottom of the outer tank, slowly decant the water from the spawning tank into the sink. When only the embryos and debris remain in a small amount of water, pour the remaining water through the embryo strainer (a plastic tri-pour beaker with the bottom removed and a piece of 500-µm mesh netting attached to the top with silicone aquarium sealer). Wet down the mesh with system water to allow the water to pass through more easily. Embryos

will catch on the mesh and smaller debris will pass through. Use a squirt bottle of system water to wash any remaining embryos from the bottom of the spawning tank into the strainer. Gently rinse the embryos on the strainer using the squirt bottle. This will push the softer remaining feces through the strainer, leaving cleaner embryos.

Turn the strainer over and gently wash the embryos into a clean plastic petri dish with the squirt bottle of system water. Fill the petri dish half way with system water to keep the embryos in water.

After spawns are taken down and embryos are harvested, wipe down the countertops and sterilize the surface with 70% alcohol.

Clean the embryos under a stereomicroscope by removing remaining feces, unfertilized eggs, and scales with a wide mouth glass pipette and a pipette pump. Unfertilized eggs can be distinguished by comparing the embryos to the stages as documented by Kimmel et al (1995). Remove embryos that are not developing normally (e.g., asymmetrical cleavage) or that do not appear to be developing (i.e., if they look like they are in the one-cell stage when all other embryos are in the 48-cell stage, it is highly likely they are unfertilized). Changing the system water after cleaning the embryos is also recommended.

Daily water renewals and removal of dead embryos are imperative for keeping embryos and larvae healthy and less susceptible to *Coleps* sp. infestations (a single cell organism that can feed on zebrafish embryos). Also limit the number of embryos to a maximum of 50 for each 100 mm petri dish; 30 embryos per dish are optimal.

Label, date, and initial the petri dishes and place the embryos in the incubator for consistent development. The incubator is set at 28.5ºC, which is used as the standard for zebrafish development (Kimmel et al. 1995). Later in the afternoon, check the embryos again to remove any missed unfertilized eggs.

If the fish are not spawning or are not producing enough embryos, refer to Maintaining a Breeding Zebrafish Colony.

Raising Larvae and Beyond

Raising zebrafish larvae is an intensive process requiring preparation and planning. At 4 days postfertilization (dpf), larvae are moved from the incubator into a lighted water bath, where they will be fed a special diet, raised in a larger volume of water, and given daily water changes. When they are large enough to regularly eat *Artemia*, they are moved to the Z-Mod.

Setup

Zebrafish larvae absorb most of their yolk by 6 dpf. However, food should be introduced to the larvae at 4 dpf to encourage a better growth rate and survival. It is best to start feeding larvae at the beginning of the week (Monday/Tuesday), when they will get better care during that critical period of development. Therefore, it is best to spawn adult fish on Wednesday to produce larvae that will be ready to be fed on Monday.

To raise larvae, prepare a water bath to maintain the temperature of the larval rearing tanks. Use a large shallow plastic container (such as an inexpensive storage tub) partially filled with system water. Use system water when filling the water bath to prevent accidental water contamination. For example, when removing the larval containers from the water bath, water could drip off the outer surface of the larval tank into another tank. Use one or two submersible heaters to maintain the water bath temperature between 26–28ºC. Keep a thermometer in the water bath to check the temperature. The heated water will increase the growth rate and provide a stable environment for the larvae. Place the water bath in a lighted area (ideally with the same 14-h light/10-h dark cycle as the adults). The light will allow the larvae to more easily detect and capture their prey.

Next, fill the larval rearing tanks, which are clear, 1-L square salad bar containers, to approximately a 2-inch depth with system water (≈500 ml). Put the larval tanks in the water bath. Adjust the water bath level so the tanks are not freely floating but the heaters are still submerged. The day before the larvae are transferred to the larval tanks, turn on the heaters so the water bath will come to the desired temperature.

When the larvae are 4 dpf, transfer them from the petri dishes in the incubator to the larval tanks. Place 20 to 25 larvae in each 1-L larval tank. Higher densities will diminish growth. To transfer the larvae from the petri dish to the tank, immerse the petri dish containing the 4 dpf larvae into the larval tank water. Remove the dish and check for any larvae still on the dish.

Label each larval container with the type of stock or transgenic line and the date of fertilization. Complete a new larvae fish feeding log sheet (Appendix C: Examples of Zebrafish Records) for each stock or transgenic line; include the parental lineage used to generate the new larvae.

Every 2–3 days refill the water bath with RO/DI water, as the water will evaporate.

Feed Schedule

Feed the dry larval food first, then the paramecia (see Paramecium Feeding), and finally *Artemia*, if needed.

Age (days)	Morning	Noon	Afternoon
1–6	Dry larval food 3,000 paramecia	Dry larval food	Dry larval food 3,000 paramecia
7–12+	Dry larval food 3,000 paramecia 3 drops *Artemia*	Dry larval food 3 drops *Artemia*	Dry larval food 3,000 paramecia 3 drops *Artemia*

Dry Larval Food

The dry food is a 1:1:1:1 mixture of Spirulina, Artificial Plankton, Micro-Food, and Cyclop-eeze. Alternatively, use Zeigler Larval AP100 (Appendix A), which is a complete mixture of dry food formulated for fish larvae. A stock of the dry food is stored in the refrigerator. As with adult fish food, larval fish food should be ordered every year to ensure

proper nutrition. For feeding, larval fish food is stored in a 50-ml conical test/centrifuge tube with an approximately 2-mm hole in the lid. Give one shake of the tube over a larval tank to dispense the dry food.

Paramecia

See Paramecium Feeding.

Artemia

At 24-h old, *Artemia* sp. are smaller and still have yolk, which is a better size for the small mouths of larval zebrafish and is nutritionally desirable. Unlike adult zebrafish, which are fed 40–48-h old *Artemia*, *Artemia* fed to larvae should be 24-h old. Therefore, an *Artemia* cone needs to be set up specifically for the larvae. Harvest and set up the larval *Artemia* cone each morning before the larvae are fed. Harvest all the 24-h old *Artemia* in the morning using the same protocol described for harvesting *Artemia* for adult zebrafish. Once diluted with system water in an evaporation bowl, the *Artemia* can be kept in the bowl for the rest of the day. There will still be eggshells and unhatched eggs in the bowl. Avoid feeding these to the larvae. Prepare the cone for the next day's *Artemia* culture using similar protocols for setting up the *Artemia* for adult zebrafish. Use only 1 L *Artemia* water (1 tbsp Instant Ocean/1 L RO/DI water) and only ¼ to ½ tsp of *Artemia* cysts (depending on number of larval tanks, ¼ tsp is sufficient to feed eight tanks).

Cleaning Larval Tanks

Start cleaning the tanks when the tank bottoms collect noticeable amounts of leftover food or on the third day of feeding (when larvae are 7 dpf). Clean the containers once a day in the morning before feeding.

Use a clean (bleach-sterilized) turkey baster to remove debris and most of the water, leaving enough water so the larvae can swim freely. Use a beaker to contain the dirty water. Be careful not to aspirate the larvae. Another method of cleaning larval tanks is to slowly pour off the water, being careful not to lose any fish. Use a clean pipette to "chase" fish away from where water is being poured off. Swish fresh system water around on the bottom of the larval tank to loosen moldy food, then pour off the fouled water.

For either method, fill the larval tank with clean system water back to a 2-inch depth or about 500 ml. Repeat cleaning if the water is still cloudy. Between each larval tank cleaning, check the dirty water in the beaker for larvae before dumping it down the sink. Empty the beaker between each tank cleaning so the origin of stray larvae is not in question. Use a clean pipette to retrieve fish.

If dead larvae are found, remove these and record the number of mortalities on the larval fish feeding log sheet (Appendix C: Examples of Zebrafish Records).

Controlling *Coleps*

The protozoan *Coleps* is ubiquitous in zebrafish colonies and typically eats decaying matter. However, large outbreaks of *Coleps* kill and eat live larvae. There are two main sources of *Coleps*: the water with the embryos and the paramecium cultures. Ideally zebrafish should not be raised in *Coleps* infested waters. To prevent *Coleps* from growing in petri dishes with the embryos, water changes should be done daily and the density of embryos should be kept low. To prevent *Coleps* from being introduced through contaminated paramecium cultures, check the cultures before feeding (see Paramecium Culture). If there is a high population of *Coleps*, it is best to raise embryos only when a clean culture can be produced.

With great care, however, it is possible to raise embryos in high *Coleps* conditions. First make a high salinity (conductivity is ≈ 2,500 µS/cm) Instant Ocean sea salt solution in a 5-gal bucket. Use this water to raise the embryos. Complete water changes are essential and should be done every day (do not wait until 7 dpf). When the larvae look like they will survive (i.e., are eating *Artemia*), slowly lower the conductivity each day by increments of 200–250 µS/cm until it is the same as the Z-Mod (1,500 µS/cm). Then the larvae can be moved to the Z-Mod.

Moving Fry to the Z-Mod

Fry are moved to the Z-Mod around day 13. They must pass an "orange belly test" wherein the larval tank is held to the light and the bellies of the fry are examined. If they are bloated and orange, this indicates they are eating *Artemia* and can be moved to the Z-Mod system. Fry should be moved to the bottom shelf of the Z-Mod, the "zebrafish nursery," where the water flow is adjusted to a slower flow rate (≈1 ml/sec).

When they are young and small (<1 month old), fry can be kept in groups of up to 20 fish per tank. As they get older and bigger, they will be separated into smaller groups of up to 15 fish per tank (see Maintaining a Breeding Zebrafish Colony).

Once the fish are introduced to the Z-Mod, they should be fed twice daily. Typically, during the first week that they are in the Z-Mod, start feeding them a tiny pinch (about the size of a grain of rice; smaller than the amount fed to adult tanks) of small, fish crumble (Bio-Oregon Bio Vita dry salmon starter that is processed in a coffee grinder so that it is small enough for the fry to eat) and 5–7 drops of *Artemia*. Increase the amount of dry salmon food and *Artemia* as they get older. The amount of food is a judgment call that relies on experience. Overfeeding can be detrimental to the fry, as leftover food can quickly be overgrown by fungus. The tanks should be checked every other day and cleaned when necessary (see Cleaning). It is best to avoid overfeeding all together by adjusting the amount of food so that the young fish are satiated, without leftover food.

Tank Outlet Size

Use the appropriate outlet with each tank. There are three types of outlets for the different stages of zebrafish life. They are color coded orange, white, and blue or red. For small fry in the nursery, use the orange outlets (mesh size 500 µm). This mesh is fine, so fry cannot pass through the holes. The mesh also catches most of the food and quickly becomes clogged; make sure the mesh is clear of debris so the tank does not overflow. When fish are slightly

bigger (≈1 month), the white outlet can be used (mesh size 1 mm). When the fish are almost adult size (≈2 months) use the blue/red outlets with the largest mesh size (4 mm). The appropriate size mesh allows for the safety of the fish and proper water flow.

Out of the Nursery

When the fish are large enough to handle a blue/red outlet (≈2 months), they are also able to deal with a stronger water flow (≈2 ml/sec). Then they can be moved from the nursery to a different shelf in the Z-mod where the flow is faster.

Paramecium Culture

Culturing paramecia (*Paramecium multimicronucleatum*) involves supporting a food chain: boiled wheat pellets are broken down by bacteria, which are eaten by flagellates, which are eaten by the paramecia. Sustaining this food chain can be very sensitive; cultures can become contaminated, paramecium populations can grow too dense and crash, or paramecium cultures can go anaerobic (and smell like rotten eggs). It is important to plan accordingly by using aseptic, sterile techniques when preparing the cultures and solutions and by checking the cultures before using them.

The general process of growing paramecia to feed larvae involves keeping stock cultures in bottles. Bottle cultures are created by inoculating a bottle of new media with an existing bottle culture of paramecia. Bottle culture propagation should continue at all times, even when no larvae are being fed. To create larger working cultures, the paramecia from the bottle cultures are used to inoculate Tupperware containers filled with culturing media. These Tupperware cultures are then fed to the larvae when their density reaches 150–200 paramecia/ml.

Prepare cultures at least 1 week (2 weeks ideally) before zebrafish larvae are fed at 4 dpf. When making cultures, stagger them by 1 week so densities do not drop while larvae are being raised. Make cultures according to how many larvae are being raised or expected. For example, when raising 16 containers of 20–25 larvae, make two Tupperware cultures (see below) 1 week before the larvae start feeding. Then 1 week later (when the larvae are feeding), make at least one more Tupperware culture.

Needed for 16 Larval Tanks

Autoclaved system water (10 L in a 25-L carboy)

Pellet stock solution (five 1-L bottles)

Paramecium bottle culture (four 1-L bottles)

Paramecium Tupperware culture

Autoclave

Autoclaving liquid takes approximately 1.5 h, plus 10 min for cooling. A few rules about the autoclave: 1) make sure all plastic and glassware are autoclavable; 2) when autoclaving

liquids, there must be an equal volume of air space as there is volume of liquid; and 3) do not tighten caps on bottles or the carboy before autoclaving.

Pellet Stock Solution

For making approximately 2 L:

- Obtain a clean 2-L glass beaker. On a hot plate, boil 1 L of RO/DI water with 20 protozoan pellets (Carolina Biological) and 2 grams of NaCl.
- Boil for 10 min and allow sediment to settle for 10 min.
- Decant supernatant into five 1-L glass bottles (≈160–200 ml per bottle).
- Top off bottles to 400 ml with RO/DI water.
- Add about 1 tsp of the remaining pellet sediment (the residue leftover after the supernatant was poured off) to each bottle. Discard remaining sediment.
- Label and date bottles.
- Autoclave pellet stock solution for 20 min.

Assessing the Purity and Density of Paramecium Cultures

For assessing the cultures:

- The purity and density of the bottle and Tupperware cultures should be assessed before using them.
- The density of the paramecia will change daily. The paramecium population will grow steadily, plateau at the highest density, then drop off. Cultures should be used at maximum density (150–200 per 1 ml).
- Use a clean, unused pipette to stir the culture.
- Remove 1 ml of the culture and observe it under the microscope.
- Slow down paramecium movement with MS-222 (tricaine).
- Under a stereomicroscope, count the number of paramecia to get an estimate of the density.
- Observe the paramecium culture under high magnification to see whether there are alien or unwanted protozoa, such as *Coleps*. If there are *Coleps* in the bottle culture, discard the culture and order more paramecia or create a new culture (see following subsection). Do not use Tupperware cultures that are infested with *Coleps*. If there is no choice and the larvae must be raised with *Coleps*, refer to Controlling *Coleps*.

Paramecium Bottle Culture

For making approximately 2 L:

- Shake an autoclaved pellet stock solution to suspend sediment.
- Aliquot 40 ml of pellet stock solution into each of four or five 1-L glass bottles.

- Add 320 ml of RO/DI water to each bottle. Label each bottle with the date the culture medium was made.
- Autoclave bottles for 20 min.
- Assess the purity and density of the existing paramecium bottle culture. The culture should be between 7–10 days old or when the density of the paramecia is the highest.
- Inoculate at least two bottles to start future paramecium Tupperware cultures. For each new bottle culture, thoroughly mix the existing bottle culture, measure out 40 ml, and add it to the new bottle culture.
- Label the bottle culture with date inoculated. Store bottle cultures at room temperature with a loose lid (paramecium cultures are aerobic).
- Ready for use in 7–10 days.

Paramecium Tupperware Culture

For making approximately 4 L:

- Make cultures at least 1–2 weeks ahead of time before raising larvae.
- Disinfect the countertop and two Tupperware containers (containers are the size of a shoebox with fitting lids, but are not air tight) by spraying them with 70% ethanol and allowing to air dry or wiping down with a clean paper towel.
- Boil 15 wheat berries per Tupperware container (30 total for 2 cultures) in RO/DI water for 7 min.
- Add 200 ml of pellet stock solution, 200 ml of inoculated paramecium bottle culture (assess the health first), and 15 drained, boiled wheat berries to 2 L of autoclaved system water for each container.
- Keep the lids on the Tupperware.
- Label the Tupperware with the date. One week later, assess the purity and density of paramecia. Note density and observations on the label.
- Tupperware cultures can be kept for up to 1 month. Check purity and density of the paramecia before feeding.

Paramecium Feeding

For feeding proceed as follows:

- Use the oldest and densest paramecium culture for feeding.
- Remove lid for only short periods, because the culture can be contaminated by airborne particles.
- Use a stainless steel spatula sterilized with alcohol or a clean pipette to stir the culture to evenly distribute the paramecia.

- Feed approximately 3,000 paramecia to each tank of larvae. Calculate the volume to feed a single tank by dividing 3,000 by the density of the Tupperware culture. Multiply the calculated volume by the number of larval tanks to get the total volume to feed the larvae. Pour the calculated total volume through the designated tight mesh paramecium net into a beaker. Measure out the volume to feed a single tank into a smaller (100 ml) beaker for each larvae tank. For example, if the paramecium density of the Tupperware culture is 100 per 1 ml, 30 ml of the culture is fed to each larval tank. If there are 8 tanks to feed, then 8 tanks × 30 ml = 240 ml. Therefore for each feeding, 240 ml of the Tupperware culture should be strained through the net and 30 ml measured out to each of the 8 tanks.
- Clean the Tupperware container with a sponge when the culture is exhausted and place it in the bleach bath.

Creating a Clean Culture

If the bottle cultures become contaminated, order new stock cultures. However, ordering new, clean cultures is not always reliable. Check incoming cultures for alien protozoa before culturing them.

Creating a pure stock of paramecia takes considerable time, but the end result is worth it. In a sterile, 6–well plate filled with sterile/autoclaved system water, transfer a single paramecium through each well using a micropipette and a clean pipette tip between each well. At the end of the wash, put the paramecium into a bottle culture with boiled wheat berries. Repeat the process with another paramecium. Add at least two cleaned paramecia to start the parent cultures. Start several parent cultures at the same time as backup. Once the parent cultures are set up, wait for them to multiply to a higher density.

Rotifers

Currently rotifers are not cultured or fed to the larvae at our facility, but they may be easier to culture than paramecia. The upsides of using rotifers are that they are direct filter feeders (and do not require a food chain as do paramecia), they can grow to a greater density than paramecia (without the population crashing), they can be collected in a 57-µm screen (and rinsed, decreasing the chance of introducing other unwanted protozoans or bacteria to the fish larvae), they can be easily fortified with Roti-Rich (Appendix A), and the protocols for rotifers are very similar to those for paramecia.

Rotifers are something to consider if or when the facility has a greater demand for larval food sources.

Maintaining a Breeding Zebrafish Colony

Maintaining the colony is mainly performed by the primary zebrafish caretaker. It involves determining when to raise a new generation for breeding, ensuring that the next generation of fish produces viable eggs for studies and future generations, and keeping a healthy, genetically diverse colony.

When and How to Breed

Zebrafish are able to breed when they are 3 months old (initial spawns can be as early as 2.5 months, with the zebrafish producing small eggs). As a general guideline, they will be able to breed until they are 1.5 years old. (Zebrafish usually live up to 2.5 years, but their gametes start deteriorating after 1.5 years.) A good breeding population should be maintained so that there are always zebrafish within breeding age.

Start a new generation of breeders every 3 to 4 months. Roughly 100 new fish should be raised each generation to maintain the breeding colony. This number can decrease or increase according to project needs. The breeding fish should always have a good age range to choose from.

To prepare for a new generation, make room on the Z-Mod, prepare paramecium Tupperware cultures, and check to see whether it is a good time for labor intensive larval rearing (e.g., larval rearing over holidays is not good timing). When starting a new generation, choose parents randomly to avoid inbreeding. Genetic diversity is important in the zebrafish colony. Set up several spawns from different tanks. When gathering each clutch or the eggs produced by a spawning group, keep the eggs separate. Raise eggs from clutches that are from strong spawners (i.e., fish that provide eggs of good quantity and quality) to be the next generation.

If the quality of zebrafish breeders starts to deteriorate, try pair-wise spawning (i.e., one male and one female). Set up many pairs and collect the clutches of eggs while keeping them separated from each tank. Assess the number of unfertilized eggs and developmental abnormalities for each clutch. Raise only the clutches that have eggs of high quality and quantity.

Labeling Generations

Labeling tanks is a somewhat arbitrary process. A good way to keep track of the generations is to color code tanks, so that a single generation will have the same colored label. Different simple names are assigned to each tank, thus a record can be kept as to which tanks have been spawned. More importantly, keep the date of fertilization on the label, so the age of the fish is readily apparent.

Grouping Fish into Z-Mod Tanks

At 3 months of age, the fish can be distinguished as male or female. Separate males and females into groups of up to 15 per 2-L tank. Keeping the sexes separate makes setting up spawns easier.

To keep reproductive hormones elevated, tanks with females require the constant presence of at least one male in the tank. When setting up female tanks, add one or two males (preferably of the same clutch) to the tank. Male tanks do not require the presence of females.

Males and females may also be grouped together. Keep in mind that small groups of six or less will lead to constant chasing, a high stress situation, and perhaps death (most likely for

the females). If there is no choice but to have a small group in a tank, place a piece of mesh in the tank to act as a hiding place for the fish to find refuge.

Factors that Affect Quality and Quantity of Eggs

Many factors affect how well zebrafish will spawn.

1. Age: Generally 6-month-old to 1-year-old zebrafish spawn the best. Animals older than 1.5 years will not spawn as many eggs or they may be of poor quality (e.g., eggs may not be viable or the number of abnormal embryos may be high).
2. How often the zebrafish are spawned:
    - Overspawning the fish decreases the quantity of the eggs in the clutch. Fish should be given at least 1 week of rest between spawning events.
    - If fish are not spawned often, eggs are reabsorbed or deteriorate in the female fish. Females should be spawned at least once a month to maintain egg quality.
3. Diet: If zebrafish do not receive the right nutrients or are not fed enough, the quality and quantity of the eggs will be affected.
4. Light cycle: A change in light cycle affects whether zebrafish spawn. It may take up to 1 week for them to acclimate to a new light cycle (e.g., daylight savings time).
5. Genetics:
    - A bottleneck in the gene pool can severely affect spawning capabilities. Outcrossing (crossing fish to a different genetic background) transgenic or mutant fish with the wild-type background (e.g., AB strain) is important to keep these lines viable. Bringing in a new source of wild-type zebrafish is a way to keep the gene pool larger (see Expanding–New Fish and Systems).
    - "Bad" genes in the gene pool can affect egg viability and egg production. This can be overcome by using the pair-wise spawning technique described above.
6. Disease: Some diseases can affect spawning. Unhealthy fish are less likely to produce viable eggs. Fish with obvious signs of disease must be removed from the system.

If there are periods of time where zebrafish are spawned infrequently, set up routine spawns to keep the female zebrafish "in shape." Keep good records of when and which females and males have been spawned. Refer to the records when setting up routine spawns, making sure to spawn females every 4–5 weeks.

Keeping a Healthy Colony

Maintaining the health of the colony is the responsibility of all zebrafish users. Unhealthy fish should be immediately removed and euthanized to reduce the chance of spreading disease to healthy fish.

Preventative measures should also be taken to keep the colony healthy. When fish are past 1.5 years in age, diseases and degeneration can become problematic. As fish age, their immune systems lose strength and diseases can appear (e.g., curled back, tumors, thin, off color,

retinal degeneration, Appendix B: General Fish Aquaculture Information). To reduce the chance of spreading diseases from older fish, fish that are more than 1.5 years old should be removed from the Z-Mod.

Euthanizing Adult Fish

A standard method of euthanizing zebrafish as described by the Institutional Animal Care and Use Committee is to use an overdose of 200–300 mg/L tricaine methanesulfonate (MS-222). To euthanize adult fish, move them from the tank or tanks into a beaker or container that contains system water with the overdose concentration of MS-222. After a few minutes, the fish will stop respirating (i.e., opercular movement will cease). Leave the fish in the solution for 10 min, then drain the water and store the fish in the freezer (in a bag or petri dish). They can be sealed in another plastic bag and placed in the refuse later.

Record of the number of fish euthanized, the tank number, and the reason for euthanasia on the mortality log sheet (Appendix C: Examples of Zebrafish Records).

Biosecurity

It is important to keep the zebrafish colony safe from pathogens. There are several control points to consider to prevent pathogens from being introduced to the colony.

1. People: Limit access to authorized personnel. This ensures no equipment is borrowed. Zebrafish users must be properly trained on the procedures (Appendix E: Training Checklist for New Zebrafish Users).
2. Fish: Know the health status of the fish. Quarantine fish obtained from outside sources (see Expanding–New Fish and Systems), do not house other fish species or aquatic organisms in the same room as the zebrafish, and bring only disinfected eggs into the facility (even these can carry disease).
3. Water: Know the source of water or make sure the filtration system is working properly, or both.
4. Equipment: Clean, maintain, and store equipment properly. Use one net per tank, and ensure that nothing is shared or borrowed.

Record Keeping

Keep records of all maintenance and feedings in the zebrafish room. If it is not written down, assume it has not been done. Filling out records is a good way to follow a checklist of what needs to be done, and it allows monitoring for sudden changes (such as water quality or a spike in mortality). Records can help prevent and solve problems (Appendix B: General Fish Aquaculture Information).

Fill out log sheets (Appendix C: Examples of Zebrafish Records) for:

- Z-Mod zebrafish feeding
- Z-Mod water quality
- Spawning record
- Larval fish feeding
- Filter change
- Mortality record
- Calibrations of pH and conductivity meters
- Chlorine and TDS test
- System water change

Zebrafish Room Power Failure Plan

An emergency plan for power failures is an important part of zebrafish colony maintenance. Every zebrafish user in our facility should be aware of this plan.

When the Power Goes Out

There is a small flashlight located inside the zebrafish room by the door.

Using the two extension cords located in the box outside the fish room, plug both extension cords into the outlets outside the south door of the freezer room. The outlets are orange, indicating they convey power from the generators.

Plug the following into the extension cords:

1. The Z-Mod is running on a power strip, located in the corner of the room to the right of the Z-Mod, southwest corner of the room.
2. Additional tanks on the shelves (if applicable) run on two power strips, which are plugged into the outlet on the west side of the room.
3. The larval tank heaters (if applicable) are on a power strip.
4. If there is still room on the extension cord, plug in the refrigerator and the *Artemia* air pump.

Perform a quick check of the system to make sure everything is running, nothing is leaking, etc.

Find the ceiling light timer, which is installed on the wall next to the light switch. Peel off the tape to open the cover. Next, remove the "on" and "off" clamps in the metal timer box, that control the ceiling lights. The metal clamps can be unscrewed and taken off the metal timer disk (it will be obvious once the box is opened). Leave the clamps inside the box. The clamps must be removed so that when the electricity does come on, the lights will not turn on when they are not supposed to. It is also important to switch the light toggle (located near the lower right corner of the timer disk) to the "off" position. It is best to have the ceiling lights off when the electricity comes back on, that way the lights will not turn on at the wrong time if electricity returns during the night.

Do not feed the fish. Get the water flowing again and cleaned up. Feeding after the system has been off for a period of time can add too much ammonia to the system.

Keep the door closed as much as possible, since the extension cords will be in the way, and to the extent possible keep the power cord connections off the floor or at least dry.

After the Power is Restored

Perform the following steps:

1. Plug all cords back into the wall sockets.
2. Correct the time in the ceiling light timer. Replace the "on" and "off" clamps to the time disk, so that the timer turns the lights on at 800 hours and off at 2200. Toggle the on/off switch located to the lower right of the time disk to "on" if the current time is between 800 and 2200 or "off" if it is between 2201 and 759 hours.
3. Double-check that everything is working properly.
4. Bundle up the extension cords and place them in the storage box outside the zebrafish room.

Expanding–New Fish and Systems

When new fish or embryos are required for an expanding zebrafish system, precautions must be taken to protect the health of the existing colony. New fish must be quarantined and only bleached embryos are allowed in the zebrafish facility.

Bringing in New Fish from a Different System

All nonembryo fish brought from another facility must be quarantined. No matter how reputable, every major zebrafish lab has or had cases of disease. It may take months for a disease to surface; so to prevent new pathogens from being introduced to the system, new fish must be quarantined. The quarantine area should be an entirely separate room away from the main zebrafish room (i.e., no adjoining entrances).

To ensure the health of the zebrafish, several rules must be followed:

1. Nothing is shared between the zebrafish room and the quarantine area. It is important that nothing goes back and forth between the two. The quarantine room is equipped and entirely independent of the zebrafish room (i.e., separate meters, nets, tanks, spawning tanks, etc.). Take only disposable ware (e.g., pipettes, tri-pour beakers) into the quarantine room.
2. Feed the quarantined fish after the zebrafish room. It is best not to reenter the zebrafish room immediately after working with the quarantined fish.
3. Wash your hands, especially after feeding or dealing with quarantined fish.
4. Be mindful. Think about what you are doing while in the quarantine area and consider yourself contaminated after you have been in the quarantine area.

When quarantined fish are spawned, the spawns must be set up in the quarantine area and the embryos must be bleached (as described in Westerfield 2007) before leaving the area.

If the original purpose of obtaining adult zebrafish is to expand the colony's gene pool or add special strains, then only the offspring of the quarantined adults can be introduced to the zebrafish room. To add progeny of quarantined adults, eggs must be bleached before being introduced to the zebrafish room (see Bringing in New Embryos from an Outside Source). Quarantined adults are never brought into the zebrafish room and must remain in quarantine for the remainder of their lives.

Setting Up and Maintaining a Quarantine Tank

Currently, two quarantine tanks are set up in the quarantine area. They are 15- and 20-gal tanks. Each has a three-filter system, a heater, and an air stone, which is connected to an air

pump with capacity for more air stones. This is not a typical household aquarium tank; the water and the environment are closely monitored and controlled.

If setting up a tank from scratch, set up the tank and allow water to go through the nitrogen cycle before adding the quarantine fish. For the quarantine tank, make system water using Instant Ocean salt and distilled water. Distilled water is used instead of the RO/DI water from the zebrafish room. This reduces chances of cross-contamination by eliminating a shared water source.

To encourage and speed up establishment of the nitrogen cycle (Figure 3 and Appendix B), seed the tank's sponge filter by adding water and bacteria from the Z-Mod's sponge filter. Next, add seed fish (fish that are no longer needed; e.g., too old to spawn) from the Z-Mod system (they will be euthanized once the quarantine fish arrive). Initially after the tank is set up and the seed fish are added, ammonia levels will be high. Do not be alarmed; it takes time for the bacteria to grow and start the nitrogen cycle. Slowly, ammonia levels should go down once the bacteria are growing. Ideally, set up the tank 4–6 weeks before the quarantine fish arrive. This should be enough time for the sponge filter to build a good population of bacteria, which will control ammonia and nitrite levels.

When the tank is initially being set up, check the pH, temperature, conductivity, ammonia, and nitrite levels daily. Add water amendments (e.g., sodium bicarbonate) and allow them to mix for at least 24 h before adding more. The goal is to get the bacteria growing and the water quality stabilized before adding the quarantine fish.

If a tank must be set up quickly, use an ammonia removing filter medium (e.g., zeolite). The drawbacks are that the water will have to be changed frequently in large amounts and the biofilter will not have a chance to grow bacteria. Maintenance tasks include:

1. Feeding (daily): First, feed the Z-mod, reserving some *Artemia* in a disposable pipette for the quarantine fish, then feed the quarantine tanks. A small container of dry salmon crumble is kept on the countertop next to the quarantine tanks. Dispose of the pipette that was used to feed *Artemia* in the quarantine room.
2. Water measurements (daily): As with the Z-Mod, water parameters of all tanks must be checked and recorded. Check the conductivity and Ammonia Alert disc once a day. Check the pH and room and water temperatures twice a day while feeding.
3. Nitrite check (weekly): Use the kit to check the nitrite levels of all tanks.
4. Water changes (when needed or weekly): To remove water from the quarantine tank, siphon off debris and water, taking care not to siphon up fish. Remove 5–10% of the tank water. To reduce cross-contamination, dissolve Instant Ocean salt in distilled water (instead of using the RO/DI water system in the zebrafish room) to the appropriate conductivity in the quarantine system water bucket. Add water amendments if needed. Add the water to the tank.
5. Water amendments (when needed): If the pH is low (<7.0), add about 5 ml of 1 molar sodium bicarbonate solution to a 25-gal tank. Add these amendments slowly to the filter so it can mix thoroughly. Ideally, the tank will be stabilized before quarantined fish are

added. Amendments can also be mixed in the quarantine system water bucket when doing water changes—this is a better and subtler change for the fish.

6. Filter changes (monthly or more depending on the number of fish): The sediment/carbon filter should be changed monthly. Do not rinse the sponge filter at the same time the sediment/carbon filter is changed. The sponge filter may be lightly rinsed if bacterial buildup decreases water flow.

Bringing in New Embryos from an Outside Source

To bring in new embryos, the eggs must be bleached before taken into the zebrafish room. Bleaching protocol is found in Westerfield (2007).

Embryos should be at least 6 h postfertilization (hpf) and no more than 24 hpf (hatched embryos cannot be bleached) for bleaching. Bleaching embryos is a delicate operation and should be done very carefully. Overbleaching can kill them.

Make a bleach solution in a 250-ml beaker by adding 100 µl of 5.25% sodium hypochlorite (bleach) to 170 ml of system water. Use a pipette to transfer up to 30 embryos to the bleach solution. Keep the embryos in the solution for 2 min while occasionally swirling the beaker to allow the entire surface area of the chorions to come in contact with the bleach solution. With a clean pipette, transfer the embryos from the bleach solution to a 250-ml beaker of clean system water. Swirl the embryos in the system water. Repeat the wash in system water one more time, then transfer the embryos back into petri dishes with fresh system water.

When the bleached embryos are 24–48 hpf, they should be manually dechorionated as bleaching will cause the chorions to become tougher. Keep in mind that even bleaching does not guarantee that no diseases will be transferred (Appendix D: Zebrafish Diseases).

Preventing the Spread of Pathogens

Adding a new aquaculture system is an opportunity to make a fresh start at establishing a colony with minimal amounts of pathogens. Since a new system will share the same space as the old one, it is highly unlikely that the new system will be kept entirely pathogen free. However, it is ideal to reduce the transfer of pathogens between systems. In the unlikely event of a disease outbreak, at least both systems will not succumb to a massive die-off or other deleterious effect.

Several things should be considered to minimize the spread of pathogens to the new system and to monitor the health of a larger colony. Typically, a new aquaculture system is seeded by transferring several tanks of healthy young fish from the old system to the new system. The introduction of fish to the new system will produce ammonia, which will start the nitrogen cycle (Figure 3) and grow the ammonia- and nitrite-metabolizing bacteria in the sponge biofilters. However, adding fish to the new system will carry over any pathogens from the old system. To eliminate transfer of pathogens to the new system, seed a system without using fish by adding large amounts of fish food to the new system daily. This should build up ammonia and start the nitrogen cycle. However, it will also take longer to achieve a good population of ammonia- and nitrite-metabolizing bacteria in the sponge biofilters without fish.

Once the biofilters are established, pathogen-free fish should be introduced to the new system. They can be grown from pathogen-free eggs. Such eggs are difficult to find, especially since not all pathogens are killed by bleach (Appendix D: Zebrafish Diseases). The Zebrafish International Research Center (ZIRC) in Oregon is working to achieve this. If pathogen-free eggs are not available, the next best step is starting with bleached eggs (see previous subsection, Bringing in New Embryos from an Outside Source).

To keep the new system free of pathogens, all zebrafish users should be familiar with ways to minimize potential pathways for pathogens to travel between the old and new systems. Sharing nonsterilized equipment and moving fish between the two systems is not allowed; these practices would guarantee the spread of pathogens. Only clean, sterilized equipment (e.g., nets, spawning tanks) can be used for both systems. Separate equipment that cannot be easily sterilized, such as meters and siphons.

Unlike a smaller colony, where more time can be devoted to monitoring the health of each tank, tracking the health of individual tanks in a larger zebrafish colony is impractical. To monitor the general health of zebrafish in a large colony, sentinel fish should be used in each system. Sentinels are fish whose sole purpose is to be sacrificed at a later date and analyzed for pathogens. There are two groups of sentinels: one group should be placed at the influent of clean water (after filtration and UV sterilization) and the other should be placed at the effluent of the waste water (before filtration and UV sterilization). The groups should each consist of approximately 10 young, pathogen–free fish (grown from the pathogen-free eggs). Every 3 months, these fish should be sent away for out-of-house pathology tests.

Conclusion

The zebrafish husbandry methods presented here are continuously evolving to meet new aquaculture technology, zebrafish capacity, spawning and breeding needs, maximum colony health, and cost-effectiveness. The success of our healthy colony relies on consistent care and a solid knowledge of fish health and aquaculture. At the NWFSC, our research using zebrafish embryos, larvae, and adults requires that no factors such as disease or pollutants confound our results. Thus we use the techniques presented here to minimize these concerns. This technical memorandum provides detailed documentation of our protocols for small-scale zebrafish husbandry. It may also be a useful reference for other facilities that have their own small-scale systems.

References

Kent, M. L., J. M. Spitsbergen, J. M. Matthews, J. W. Fournie, and M. Westerfield. 2007. Diseases of zebrafish in research facilities. Zebrafish International Resource Center (ZIRC) Health Services Zebrafish Disease Manual. Online at http://zebrafish.org/zirc/health/diseaseManual.php [accessed 10 April 2009].

Kimmel, C. B., W. W. Ballard, S. R. Kimmel, B. Ullmann, and T. F. Schilling. 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203:253–310.

Westerfield, M. 2007. The zebrafish book: A guide for the laboratory use of zebrafish (*Danio rerio*). 5th edition. University of Oregon Press, Eugene.

Appendix A: Ordering Information

Fish Food

Brine Shrimp Direct, Ogden, Utah: *Artemia* nauplii premium grade, Spirulina. Online at www.brineshrimpdirect.com [accessed 17 April 2009]; phone (800) 303-7914.

Bio-Oregon, Longview, Washington: salmon starter–Bio Vita #2. Online at http://www.bio-oregon.com/ [accessed 17 April 2009]; phone (800) 962-2001.

Carolina Biological Supply Company, Burlington, North Carolina: paramecia (*Paramecium multimicronucleatum)*, protozoan pellets. Online at: www.carolina.com [accessed 17 April 2009]; phone (800) 334-5551.

General Fish and Aquarium Supplies

Drs Foster & Smith, Rhinelander, Wisconsin: conductivity meter, solutions, Instant Ocean, general aquarium and fish supplies. Online at www.DrsFosterSmith.com [accessed 17 April 2009]; phone (800) 381-7179.

That Pet Place, Lancaster, Pennsylvania: general fish supplies, test kits, Ocean Clear filters, Micro-Food, Cyclop-eeze. Online at www.thatpetplace.com [accessed 17 April 2009]; phone (888) 842-8738.

Aquatic Eco-Systems Inc., Apopka, Florida: general fish supplies, *Artemia* cones, Artificial Plankton, Larval Diet AP100, Reef Carbon, Roti-Rich. Online at www.aquaticeco.com [accessed 17 April 2009]; phone (877) 347-4788.

PetStore.com, Garden Grove, California: Ammo-Lock, Net Soak. Online at http://www.petstore.com/ps_ViewItem-idproduct-AP8173.html [accessed 18 May 2009]; phone (888) 993-2378.

Zebrafish (*Danio rerio*) Hardware

AquaFX, Winter Park, Florida: RO/DI system and filters. Online at www.aquariumwaterfilters.com [accessed 17 April 2009]; phone (877) 256-3467.

Emperor Aquatics Inc., Pottstown, Pennsylvania: UV light bulb and UV sterilizer parts. Online at http://www.emperoraquatics.com [accessed 17 April 2009]; phone (610) 970-0440.

AZPonds and Supplies Inc., Birdsboro, Pennsylvania: alternative source for UV light bulb and parts. Online at http://www.azponds.com/uv_parts.htm [accessed 17 April 2009]; phone (800) 722-8877.

Marine Biotech, Beverly, Massachusetts (recently acquired by Aquatic Habitats): Z-Mod system and tanks. Online at http://www.marinebiotech.com/ [accessed 17 April 2009]; phone (888) 624-8265.

Thoren Aquatics Systems Inc., Hazelton, Pennsylvania: spawning cages and lids. Online at http://thorenaquatics.com/index.htm [accessed 17 April 2009]; phone (570) 454-3517.

Cambro Manufacturing Company, Huntington Beach, California: larval tanks. Online at www.cambro.com/ [accessed 17 April 2009]; phone (800) 833-3003.

Appendix B: General Fish Aquaculture Information

Below is background material on fish aquaculture to help understand why the Northwest Fisheries Science Center zebrafish (*Danio rerio*) facility uses the protocols described in this tech memo.

The basics of fish physiology:

1. Cold-blooded: Temperature affects the fish's immune function and metabolic rates (e.g., high temperature increases the demand for oxygen, food, and growth).
    - High temperatures may cause stress and death.
    - Low temperatures may cause slower growth and sometimes death.
2. Osmotic regulation: With freshwater fish, water moves into the fish. The kidneys excrete high volumes of dilute urine and the skin is a barrier to the uptake of water. With pathogens or rough handling, the kidneys must work harder (sometimes leading to kidney failure).
3. Gills: The essential functions of the gills are respiration, excretion of nitrogenous waste (ammonia), and ion exchange. They are especially vulnerable to various forms of damage such as from bacterial or fungal infections.

Important water considerations:

1. Dissolved oxygen (DO): As water temperature increases, DO decreases.

Temperature (°C)	O_2 at 100% saturation (mg/L)
5	12.76
10	11.28
15	10.07
20	9.08
25	8.24
30	7.54

As temperature increases, the oxygen requirement (metabolism) of the fish increases.

The movement of water (e.g., via air stones) will keep oxygen in the water. The factors that affect DO are the air-water interface, utilizers of oxygen (fish), and producers of oxygen (mechanical or photosynthetic).

2. Temperature: The optimum temperature for zebrafish is approximately 26°C. As mentioned previously, temperature can affect DO, and sudden changes in temperature and DO can be a stressor.

3. Source of the water: It is necessary to filter incoming water for particles and contaminants (such as chlorine).
4. Hardness and alkalinity: Hardness is the amount of cations in the water and can have interactions with other water parameters or disease treatments. Alkalinity is the buffering capacity of the water (resists changes in pH) and is related to the amount of bicarbonate in the water.
5. The nitrogen cycle (Figure 3): Ammonia and nitrite are toxic to the fish; nitrate is toxic at very high concentrations (which is why water changes are important). Nitrosomonas and nitrobacter—bacteria that convert ammonia and nitrite—remove the buffering capacity (alkalinity) so the pH will naturally go down. Under low pH conditions, ammonium (NH_4+) will accumulate, as the nitrosomanas cannot convert this ion. An increase in pH will cause nontoxic ammonium to convert to toxic ammonia. The nitrogen cycle (nitrosomonas and nitrobacter) takes time to establish (4–6 weeks). The reaction is aerobic, so the biological filter must not become anaerobic. Also the bigger the surface area of the filter, the higher the fish capacity the system can support.
6. Filtration: This is required for closed systems. There are three parts to filtration.
    - Biological filters grow bacteria for the nitrogen cycle. Sudden changes in water quality, therapeutics, and antibiotics can destroy the biofilter. Examples of biofilters are foam, floss, and gravel (they provide surface area for bacteria).
    - Mechanical filtration removes particulate matter. Examples are foam, filter floss, and gravel (1/4–1/8 inch diameter).
    - Chemical filters remove small molecular weight compounds. Examples are activated carbon, dolomite, zeolite, ion exchangers, and peat moss (but it also lowers pH).
7. Water sterilization: It is important to remove pathogens from the water source in flow through systems or in closed recirculating systems. Water should be UV or ozone sterilized.
8. Water changes: They are helpful in diluting waste products (e.g., nitrate) and correcting the pH level.
9. Carrying capacity of the system: The number of fish sustainable in a closed system is determined by the amount and capabilities of filtration.
10. Chlorine (and chloramines): It is toxic to fish. Chlorine can be removed by sodium thiosulfate, allowed to dissipate with time, aeration or agitation (to speed release), or with an activated carbon filter.
11. Other toxicants: Toxicants such as pesticides, fertilizers, metals, gases, and smoke affect the health of the fish.
12. Change is not good: Sudden changes in water quality stress the fish and can have detrimental effects on biological filters.

Appendix C: Examples of Zebrafish Records

What follows are examples of forms used in record keeping for the Northwest Fisheries Science Center Zebrafish (*Danio rerio*) colony. They are:

- Z-Mod Zebrafish Feeding
- Z-Mod Water Quality
- Spawning Record
- Larval Fish Feeding
- Filter Change
- Mortality Record
- Calibration of pH and Conductivity Meters
- Chlorine and total dissolved solids (TDS) Tests
- System Water Change

Z-Mod Zebrafish Feeding

Month/year: _____

	AM				PM				
Date	SS	A	Time	Initials	SS	A	Time	Initials	Notes:
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									
16									
17									
18									
19									
20									
21									
22									
23									
24									
25									
26									
27									
28									
29									
30									
31									

Z-Mod Water Quality

Month/year: _____

	AM			PM							
Date	Room temp	H_2O temp	pH	Room temp	H_2O temp	pH	Conduct.	NH_3	NO_2	RO/DI H_2O added	Comments:
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
14											
15											
16											
17											
18											
19											
20											
21											
22											
23											
24											
25											
26											
27											
28											
29											
30											
31											

Spawning Record

Date spawned	Males		Females		Spawning time (approx.)	Study	No. of eggs (approx.)	Initials	Notes:
	#	Tank name	#	Tank name					

(Form extends to fill one page.)

Larval Fish Feeding

Fill out one sheet per zfish strain/stock

Tank # :_____

Parent#: _____

Date Fertilized: _____

	AM					Noon				PM						
Date	Dry	Para.	A	Time	Initial	Dry	A	Time	Initial	Dry	Para.	A	Time	Initial	Water change	Notes:

(Form extends to fill one page.)

Filter Change

Once a month or depending on density of fish in system and psi
Follow instructions in zebrafish maintenance guide.

Date	Initials	Pressure	Filter #	Notes:

(Form extends to fill one page.)

Mortality Record

Date	Tank	# Dead	Notes:

(Form extends to fill one page.)

Calibration of pH and Conductivity Meters

Once a month
Follow instruction manuals for each meter.

Date	Initials	pH	Conductivity	Notes:

(Form extends to fill one page.)

Chlorine and TDS Tests

Once a month
Follow instruction manuals for each test kit. Test chlorine levels in Z-Mod. Test TDS of the RO/DI water.

Date	Initials	Chlorine	TDS	Notes:

(Form extends to fill one page.)

System Water Change

To be changed every 1–2 weeks
Turn off pump. Empty out sump to above the drain. Fill sump with system water from the 50-gallon tank. Check ammonia levels and conductivity after 3 hours.

Date	Initials	Notes:

(Form extends to fill one page.)

Appendix D: Zebrafish Diseases

Zebrafish (*Danio rerio*) are susceptible to many diseases. Transmission among fish can happen quickly, especially when they are in an unnatural, stressful, and crowded environment. Understanding types of diseases, how they are transmitted, and steps to prevent outbreaks can keep the fish healthy. This is a brief introduction to zebrafish disease, diagnosis, and prevention. For a more extensive review, see the Zebrafish International Research Center (ZIRC) Health Services Zebrafish Disease Manual (Kent et al. 2007).

Common Diseases of Zebrafish

The majority of bacteria, fungi, and parasites that cause disease in aquaculture and in nature are opportunistic pathogens that are ubiquitous in aquatic environments. Stress from transport, endocrine changes (e.g., spawning, aging), handling, crowding, and dramatic fluctuations in water quality may trigger disease outbreaks.

Bacterial Disease, Mycobacteriosis

Forty percent of zebrafish facilities are infected (ZIRC).

It causes serious diseases, tuberculosis and leprosy.

It is widespread and ubiquitous in aquatic ecosystems. The bacteria colonize asymptomatically, with chronic and acute infections.

Conditions that encourage mycobacteria growth are low DO, low pH, warm water, high organic load, and clean water (less competition).

It survives in surface biofilms (tank surface), which harbor and protect mycobacteria.

Signs of mycobacteriosis:

- External signs: swollen belly, skin ulcers, exopthalmia (pop eye), and general malaise.
- Internal signs (target tissues): granulomas throughout viscera, swollen spleen (sometimes gone), kidney, and liver.

Other target tissues affected less often are gills, skin (unless visible lesions), muscle, nervous tissues, and the gut (contains other bacteria).

Infections are notoriously difficult to treat because mycobacteria are naturally resistant to antibiotics.

Protozoan Parasite, Microsporidia (*Pseudoloma neurophilia*)

Fifty-nine percent of facilities are infected (ZIRC).

Signs of microsporidia:

- In adults and juveniles, chronic infections cause emaciation and skeletal deformities (skinny fish disease), reduced growth, and fecundity problems.
- In larval fish, acute infections cause mortality.

Primary sites of infection are the spinal cord and the hindbrain.

Its life cycle takes place intercellularly and produces infectious spores.

Infectious spores within the eggs may not be killed by chlorine treatments.

It can be treated with fumagillin, an antibiotic taken orally.

Noninfectious Diseases

Nutritional imbalances:

- Vitamin E/selenium deficiency causes degenerative muscle disease.
- Vitamin C deficiency (also called broken back disease) causes scoliosis (misshapen vertebrae at major pressure points on spinal column). Vitamin C participates in conversion of cartilage to bone.

Methemoglobinemia (nitrite toxicity) or "brown blood disease" is caused by excess nitrite binding to hemoglobin (inhibiting the ability of blood to carry oxygen). This is a reason why water quality must be monitored. To treat the toxicity, add NaCl (the chloride will reverse the reaction), stop feeding, and determine why nitrite levels are high.

Ammonia toxicity or "callus and blister" causes a proliferation of cells and an accumulation of fluid in the gill. At low concentrations, it causes a diffuse gill epithelial tissue proliferation. At high concentrations, a typical response to the toxin in the water is lifting of the gill epithelium from the supporting structure.

Nephrocalcinosis is the formation of calcium in kidneys, which indicates too much calcium in the water. Mineralization on kidney tubules is associated with high concentrations of dissolved carbon dioxide in water or excessive calcium or magnesium in the diet. It is important to use sodium bicarbonate—not calcium carbonate—when buffering fish water.

Gill hyperplasia is associated with poor water quality such as active chlorine, excess metals such as copper and zinc, or excess ammonia particulates.

Gas bubble disease is caused by supersaturated concentrations of gases, photosynthesis, imperfections in plumbing fixtures, or rapid heating of water.

Egg associated inflammation and fibroplasia (EAIF) occurs when females retain eggs, which degenerate and eventually push out the abdominal wall. EAIF is very common and often associated with secondary bacterial infections, including mycobacteriosis. The exact cause is unknown.

Routes of Disease Transmission

Direct transmission: Pathogens can pass between live fish. This is why fish coming from other facilities must be quarantined and why equipment must not be shared between fish facilities or individual tanks. Direct transmission also occurs when fish eat the infected tissues of other fish; therefore, it is important to remove sick and dead fish.

Vertical transmission: Pathogens can be passed from the mother into the egg. Since the pathogen is in the eggs, bleaching cannot kill all pathogens.

Biofilm transmission: Biofilm is the layer of material that builds up on the inside of tanks. Pathogens can grow and multiply in biofilm.

Aerosol transmission: Some pathogens are small enough to drift in the air, which is why it is important to put lids on the tanks.

Diagnostic Techniques

It is imperative to diagnose disease outbreaks as soon as possible, so a proper treatment can be quickly implemented. Diagnoses can be done in-house or be sent to ZIRC (http://zebrafish.org). Samples can be shipped to ZIRC and technicians can perform the techniques described below. They are experienced and reliable at identifying pathogens.

Wet mounts are easy and can be done in-house. The best results are found on freshly dead fish. To take wet mounts, place the tissue sample in a drop of water on a microscope slide and cover slip. Examine at all powers of magnification, looking for external parasites (e.g., protozoans and monogeneans), bacteria (e.g., Flavobacterium, a long flexing rod-shaped bacterium), and fungi (e.g., Saprolegnia). Take wet mounts of the external and internal parts of the fish.

- External exam: Look for behavioral signs, body condition, swollen abdomen, and abnormal growths (e.g., tumors). Photograph any gross abnormalities. Tissues to sample and wet mount:
    - Gills, examine the individual filaments.
    - Fins, examine the end of the pectoral fin.
    - Skin scraping, use a cover slip to scrape behind the pectoral fin and any external lesions (on the skin, fin, or gills).
- Internal exam: Disinfect the external surface (with ethanol). To avoid contamination, aseptically open the abdomen with sterile instruments and avoid cutting the digestive tract (do not release the gut flora). Do a gross examination of organs. Tissues to sample and examine microscopically:

- Lower intestine, chop up and wet mount on to a slide.
- Brain, smear and wet mount on to a slide.
- Contents of any cysts (helminthes and sporozoa).

Bacterial culture should be done with the bacteria from the posterior kidney (bacteria are more likely to be here) and any visible lesions on the organs. Using aseptic techniques, collect kidney tissue from a small fish by entering the fish from the dorsal side downward to the kidney. Culture the bacteria on the appropriate agar.

Viral culture can be done by collecting selected internal organs (usually kidney, spleen, and liver) and inoculating the appropriate fish cell culture. Use aseptic techniques.

Histopathology can be performed by removing pieces of organs (tissue size no greater than 1 cm for rapid fixation) and placing them in a fixative such as Dietrich's fixative (use a fixative volume of 10:1 fixative:tissue). Dietrich's fixative is used because it softens scales, decalcifies bone, and provides good nuclear staining. Storage in plastic jars is also suggested, especially if the samples will be shipped.

Treatments and Controls

Below is a brief overview of the treatments commonly used to combat diseases in aquaculture. However, make sure to obtain an accurate diagnosis before proceeding with a treatment, because an incorrect treatment could harm the fish.

External treatments are generally needed for external pathogens. The treatment compound is added to the water.

- Types of treatment and duration of dose:
  - Dip is for a short time at a relatively high concentration.
  - Bath is a longer time at a lower concentration.
  - Prolonged treatment is over a long duration (the compound decomposes with time).
- Formalin treatments must have adequate aeration and proper storage (above 40°F or else it turns into paraformaldehyde). Formalin is caustic.
- Copper sulfate is an algaecide that is effective against some external parasites. Toxicity increases with low alkalinity (toxic at alkalinity < 50 mg/L).
- Potassium permanganate is a strong oxidizing agent that is effective against some fungal and bacterial diseases. The dose is related to the amount of organic matter in the water.
- Sodium chloride causes osmotic shock on external parasites. The salt is also a general stress alleviator for freshwater fish (the elevation of salinity reduces osmotic stress on fish).

Internal treatments are generally needed for internal pathogens. The treatment compound (typically antibiotics) must get inside the fish (e.g., via the feed). To treat with antibiotics, there

must be a diagnosis that includes the resistance pattern of the bacterial pathogen. Since the most common dosing method is via the feed, the treatment must start in timely manner (e.g., fish must be feeding). Feed for the entire duration of the treatment.

Eradication is the depopulation of the facility and chlorination of the system (a fresh start). Exhaust all other possibilities before eradicating the system. This treatment is the last resort when a disease outbreak cannot be controlled. Eradicating the system means destroying all fish and sterilizing the system to wipe out the disease. This will also destroy the biofilters, meaning the entire system will have to be reseeded with new fish or fish grown from embryos to repopulate the system.

Prevention

Remember that pathogens are ubiquitous in aquaculture and fish can live with a level of pathogens. Keeping fish healthy and eliminating the introduction of new pathogens are the best ways to prevent disease outbreaks. Use the following steps to prevent an outbreak:

- Quarantine incoming fish.
- Maintain equipment (e.g., UV system) and make sure all equipment is working properly.
- Remove sick and dead fish immediately.
- Keep good records of mortality and water quality (to look for patterns or sudden changes).
- Properly clean, disinfect, and store nets and equipment (to prevent tank-to-tank spread).
- Do not share equipment between aquaculture systems or within the aquaculture system (e.g., do not use the same net between tanks without sterilizing it).

Zoonosis

Human infections are rare (usually only in people who are immune compromised). Some strains of mycobacteria can cause “fish handler’s disease.” Hand washing and good hygiene are the best prevention techniques.

Appendix E: Training Checklist for New Zebrafish Users

Use this checklist as a guide when introducing new zebrafish (*Danio rerio*) users to the zebrafish room.

☐ General room set up
- –RO/DI versus system water
- –How the Z-Mod works: plumbing, filters, sump, etc.
- –Location of nets, tanks, logbook, food, etc.
- –Location of the procedure book
- –Location of emergency plan

☐ Biosecurity/health management
- –Hand washing
- –Quarantine rules
- –No sharing equipment
- –No outside equipment
- –Bleach/sterilize everything that comes in contact with fish between uses
- –No chemicals/toxins in the fish room

☐ Making system water

☐ Spawning procedures
- –Records: location of spawn logbook and log sheet (on wall), refer to it before setting up a spawn (do not use fish that have been spawned within 1 week), good for future reference
- –Spawning cycle: set up spawns in evening, spawn occurs about 3 h after lights come on in the morning
- –Light cycle is important: on from 800 hours to 2200, changes in cycle will affect spawning
- –Tanks and lids
- –Organization of zebrafish: male and female tanks, female tanks have one male to keep females fertile, mutants and transgenic strains are mixed sexes
- –How to sex a fish
- –Nets: one net per tank, rinse net after use, Net Soak solution
- –Use black mesh in tanks with less than six fish; this prevents fighting among fish

☐ Collecting eggs
- –Put fish back into correct tanks, then back on the Z-Mod
- –Use strainer, rinse eggs, wash into a petri dish
- –Sort eggs

☐ How to recognize fertilized versus unfertilized eggs

☐ Feeding the fish
- –Typical weekday feeding
  - –two times/day (morning and afternoon)
  - –salmon starter and *Artemia*
- –Typical weekend feeding:
  - –feeding
  - –water quality
  - –start new *Artemia*
  - –fill sump with RO/DI water
  - –larvae feeding if applicable
- –Harvesting *Artemia*: two-cone system (0–24 h and 24–48 h), allow shrimp to completely settle (≈5 min), use spigot to drain 24–48-h *Artemia* into net, rinse off salty water, dilute *Artemia* into bowl, drain 0–24-h *Artemia* into 2-day-old cone via spigot
- –Setting up new *Artemia*: scrub cones with a sponge between uses, *Artemia* water, placement of aeration hose

☐ Larvae care and feeding
- –Larvae set up–water bath, temp at ≈28°C
- –Water changes every morning before feeding
- –Feeding schedule: dry food, paramecia, 24-h *Artemia* (at 7 dpf+)
- –Larvae *Artemia* cone
- –Larval feeding log sheet

Recent NOAA Technical Memorandums

published by the
Northwest Fisheries Science Center

NOAA Technical Memorandum NMFS-NWFSC-

99 Rawson, K., N.J. Sands, K.P. Currens, W.H. Graeber, M.H. Ruckelshaus, R.R. Fuerstenberg, and J.B. Scott. 2009. Viability criteria for the Lake Ozette sockeye salmon evolutionarily significant unit. U.S. Dept. Commer., NOAA Tech. Memo. NMFS-NWFSC-99, 38 p. NTIS number pending.

98 Johnson, L.L., G.M. Ylitalo, M.S. Myers, B.F. Anulacion, J. Buzitis, W.L. Reichert, and T.K. Collier. 2009. Polycyclic aromatic hydrocarbons and fish health indicators in the marine ecosystem in Kitimat, British Columbia. U.S. Dept. Commer., NOAA Tech. Memo. NMFS-NWFSC-98, 123 p. NTIS number pending.

97 Roegner, G.C., H.L. Diefenderfer, A.B. Borde, R.M. Thom, E.M. Dawley, A.H. Whiting, S.A. Zimmerman, and G.E. Johnson. 2009. Protocols for monitoring habitat restoration projects in the lower Columbia River and estuary. U.S. Dept. Commer., NOAA Tech. Memo. NMFS-NWFSC-97, 63 p. NTIS number pending.

96 Currens, K.P., R.R. Fuerstenberg, W.H. Graeber, K. Rawson, M.H. Ruckelshaus, N.J. Sands, and J.B. Scott. 2009. Identification of an independent population of sockeye salmon in Lake Ozette, Washington. U.S. Dept. Commer., NOAA Tech. Memo. NMFS-NWFSC-96, 18 p. NTIS number PB2009-108060.

95 Harms, J.H., J.A. Benante, and R.M. Barnhart. 2008. The 2004–2007 hook and line survey of shelf rockfish in the Southern California Bight: Estimates of distribution, abundance, and length composition. U.S. Dept. Commer., NOAA Tech. Memo. NMFS-NWFSC-95, 110 p. NTIS number PB2009-106740.

94 Fleischer, G.W., K.D. Cooke, P.H. Ressler, R.E. Thomas, S.K. de Blois, and L.C. Hufnagle. 2008. The 2005 integrated acoustic and trawl survey of Pacific hake, *Merluccius productus*, in U.S. and Canadian waters off the Pacific coast. U.S. Dept. Commer., NOAA Tech. Memo. NMFS-NWFSC-94, 41 p. NTIS number PB2009-104774.

93 Keller, A.A., B.H. Horness, E.L. Fruh, V.H. Simon, V.J. Tuttle, K.L. Bosley, J.C. Buchanan, D.J. Kamikawa, and J.R. Wallace. 2008. The 2005 U.S. West Coast bottom trawl survey of groundfish resources off Washington, Oregon, and California: Estimates of distribution, abundance, and length composition. U.S. Dept. Commer., NOAA Tech. Memo. NMFS-NWFSC-93, 136 p. NTIS number PB2008-113766.

92 Levin, P.S., M.J. Fogarty, G.C. Matlock, and M. Ernst. 2008. Integrated ecosystem assessments. U.S. Dept. Commer., NOAA Tech. Memo. NMFS-NWFSC-92, 20 p. NTIS number PB2008-113765.

Most NOAA Technical Memorandums NMFS-NWFSC are available online at the Northwest Fisheries Science Center web site (http://www.nwfsc.noaa.gov).